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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12Q 1/68

A3

(11) International Publication Number: WO 00/28081

(43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/IB99/01958

(22) International Filing Date: 9 November 1999 (09.11.99)

(30) Priority Data:
60/107,293 9 November 1998 (09.11.98) US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
3 August 2000 (03.08.00)

(54) Title: RESTRICTED AMPLICON ANALYSIS

(57) Abstract

The present invention generally provides a method which facilitates the detection of polymorphisms (or mutations). The method is directed to the analysis of so–called endonuclease site polymorphisms (ESPs) that result in the gain or loss of a restriction endonuclease site. In essence, the ESP is probed with the restriction endonuclease reagent prior to amplification, whereby amplification is prevented and consequently no signal is observed when cleavage takes place. Unambiguous allele calling is performed by comparing the signals obtained with and without cleavage with the restriction endonuclease reagent. The method is particularly useful for multiplex genotyping, involving the parallel analysis of large numbers of single nucleotide polymorphisms. Preferred methods for detecting the amplicons involve hybridization to an arrayed or otherwise identifiable set of cognate probe fragments or oligonucleotides.

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/IB 99/01958

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 34015 A (PENN STATE RES FOUND) 18 September 1997 (1997-09-18) the whole document ---	1-24
X	WO 98 12352 A (GEN HOSPITAL CORP ;UNIV LELAND STANFORD JUNIOR (US)) 26 March 1998 (1998-03-26) the whole document --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	14/06/2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/IB 99/01958

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

Internal Application No
PCT/IB 99/01958

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RESTRICTED AMPLICON ANALYSIS

Field of the Invention

The present invention generally provides a method which facilitates the detection of polymorphisms (or mutations). The method is directed to the analysis of so-called endonuclease site polymorphisms (ESPs) that result in the gain or loss of a restriction endonuclease site. In essence, the ESP is probed with the restriction endonuclease reagent prior to amplification, whereby amplification is prevented and consequently no signal is observed when cleavage takes place. Unambiguous allele calling is performed by comparing the signals obtained with and without cleavage with the restriction endonuclease reagent. The method is particularly useful for multiplex genotyping, involving the parallel analysis of large numbers of single nucleotide polymorphisms. Preferred methods for detecting the amplicons involve hybridization to an arrayed or otherwise identifiable set of cognate probe fragments or oligonucleotides.

Background of the Invention

Molecular approaches for genetic analyses trace the nucleotide sequence variation that occurs naturally and randomly in the genomes of all living species. Knowledge of the DNA polymorphisms among individuals and between populations is important in understanding the complex links between genotypic and phenotypic variation. In the absence of complete data about sequence variation, one relies on the ability to identify 'nearby' markers that allow to infer the location of certain relevant loci or causal sequence variations. The informativeness of the marker depends on the magnitude of the linkage disequilibrium. Markers can be used in linkage studies to search for candidate genes and in association studies to identify the functional allelic variation on candidate genes that influence inter-individual variation.

The vast majority of sequence variation consists of nucleotide substitutions, often referred to as single nucleotide polymorphism's (SNPs), resulting from mutations that have accumulated during evolution. Most of these nucleotide

changes are genetically silent; i.e., they have no measurable biological effect, but provide an immense reservoir of variation in DNA structure. Most methods for genetic analysis used today rely on the detection of nucleotide sequence variation which can be measured by DNA fragment analysis using electrophoretic separation, in which DNA fragments are fractionated based on size or conformation. Occasionally the nucleotide sequence variation will affect either the presence of the DNA fragment or its mobility. In this way the primary nucleotide sequence variation will give rise to easily detectable DNA fragment polymorphism. Since polymorphic DNA fragments are derived from precise locations on the organism's genome, they can serve as reliable genetic markers, or landmarks to identify and locate genes.

A host of assays to detect DNA polymorphisms, and SNPs in particular, have been developed. In some of these assays (*e.g.*, RFLP [Botstein, D., White, R.L., Skolnich, M., Davis, R.W., *Am. J. Hum. Genet.* 32:314-331 (1998)], CAPS [Konieczny, A. Ausubel, J.F., *Plant J.* 4:403-410 (1993)], dCAPS [Neff, M.M. Neff, J.D., Chory, J., Pepper, A.E., *The Plant Journal* 14:387-392 (1998)], PIRA [Steinborn, R., Muller, M., Brem, G., *Biochim. Biophys. Acta* 1397:295-304 (1998)]), restriction enzymes are used to detect polymorphic nucleotide sequences that affect cleavage. The specificity of restriction enzymes is such that they exhibit a unique sensitivity to detect single nucleotide differences occurring in their recognition sites. The principal strengths of restriction enzyme-based genetic analyses are the ease of use and the robustness of the assays. In the majority of the cases, the restriction site polymorphism is used to detect known, previously identified SNPs and the assay consists of any electrophoretical fragment analysis. In one report, the allelic variation is detected in a solid-phase ELISA-type setting [Truett, G.E., Walker, J.A., Wilson, J.B., Redmann, S.M. Jr., Tulley, R.T., Eckardt, G.R., Plastow, G., *Mamm. Genome* 9:629-632 (1998)].

In WO 91/17269, Lerner *et al.* describe a different method for mapping a eukaryotic chromosome by restriction endonuclease mapping of discrete DNA sequences which are complementary to a region of a eukaryotic chromosome.

Vos *et al.*, *Nucl. Acids Res.* 23:4407-4414 (1995) and EP 0 534 858 describe a technique for DNA fingerprinting called AFLP which is based on the

selective polymerase chain reaction based application of restriction fragments of a digest of genomic DNA. The application reaction depends on the use of primers that extend into restriction fragments amplifying only those fragments in which prior extensions match the nucleotide sequence flanking the restriction sites.

Another method utilizing DNA amplification steps is set out in Williams *et al.*, *Nucl. Acids Res.* **18**:6531-6535 (1990), who describe a DNA fingerprinting method termed random amplified polymorphic DNA.

DNA amplification fingerprinting was described by Caetano Anolles in *Bio/Technology* **9**:553-557 (1991). Still another fingerprinting technique called arbitrarily primed PCR was described in Welsh *et al.*, *Nucl. Acids Res.* **18**:7213-7218 (1990) and Welsh *et al.*, *Nucl. Acids Res.* **19**:861-866 (1991).

In WO 94/11530, Cantor *et al.* describe materials and methods for position and sequencing by hybridization. Cantor *et al.* also describe methods for creating assays of DNA probes useful in the practice of their method.

The major shortcoming of the current methods of genetic analysis is the limited resolution of the DNA fragment analysis systems, namely the number of DNA fragments that can be separated in a single assay. Generally the fractionation resolution ranges from tens to a couple of hundred DNA fragments, at the most. Consequently, current genetic analysis methods are limited to a few hundred to a thousand genetic markers. While this resolution has been sufficient for analyzing simple genetic traits determined by single genes, the analysis of complex traits, which is now being undertaken and which involve general or many different genes, will require the analysis of a much larger number of genetic markers. It is anticipated that such studies will require from a few thousand to possibly several hundred thousand genetic markers. Although this could conceivably be accomplished by performing many parallel assays, such scaling up will be cost- and labor prohibitive.

A technology that has great potential and which is generating widespread interest in the so-called micro-array technology (DNA chips). In general, these methods are based on measurement of the hybridization of DNA sequences in solution to probe sequences that are arrayed on a solid surface. When assaying nucleotide polymorphisms, the detector relies on the small differences in hybridization efficiency

between two different DNA sequences. In one format, fluorescently labeled sample DNA is hybridized to dense arrays of probe nucleic acids, sequence-specific hybridization signal is detected by scanning confocal microscopy, and DNA variants scored as (predictable) differences in the hybridization pattern. The micro-arrays are fabricated either by in-situ light-directed oligonucleotide synthesis [Fodor, S.P.A. *et al., Science* **251**: 767 (1991)] or by spotting DNA (off-chip synthesized oligonucleotides or PCR fragments) in an automated procedure. The technology has already been demonstrated in the scoring of mutations in mitochondrial DNA [Chee, M. *et al., Science* **274**: 610-614 (1996)], the HIV genome [Lipshutz, R.J. *et al., Biotechniques* **19**: 442-447 (1995)], the CFTR cystic fibrosis gene [Cronin, M.T. *et al., Human Mut.***7**: 244-255 (1996)], the BRCA1 breast cancer gene (Hacia, G.H. *et al., Nat. Genet.* **14**: 441-447 (1996)] as well as the entire yeast genome [Winzeler, E.A. *et al., Science* **281**:1194 (1998)]. In comparison with most other assays, micro-arrays provide a platform for high-throughput, massively parallel polymorphism detection.

A major disadvantage with the use of microarrays relates to the complexity of the hybridization reaction. The detection relies on the very small difference in hybridization of DNA sequences differing by only one nucleotide. In general, a set of 4 oligonucleotides, differing only in the identity of the central base, is synthesized for each position in the target sequence that has to be interrogated. In practice, the number of oligonucleotides needed to correctly genotype one SNP is much larger, involving up to 56 different oligonucleotides spanning the variable base [Wang *et al., Science* **280**: 1077-1082 (1998)]. The degree of redundancy is also dramatic if one wants to screen the target DNA for all possible mutations; the design then includes overlapping oligonucleotide-sets that are offset by one base (a process known as tiling). It should be noted that the detection of SNPs by hybridization to arrays depends on the use of short oligonucleotide probes. With longer probes such as DNA fragments in the size range of 50 to 500 base pairs or larger, it is not possible to distinguish the SNP alleles.

Summary of the Invention

The present invention is directed to methods for genotyping polymorphisms that result in the gain or loss of an endonuclease cleavage site. Such polymorphisms are referred to hereinafter as endonuclease site polymorphisms (ESPs). Polymorphisms detectable according to the methods of the present invention include single nucleotide polymorphisms (SNPs). The methods of the present invention exploit the high discriminatory power of restriction enzymes in a "Restricted Amplicon Assay" (RAA) which generally comprises the following steps (*see* Figure 1):

(a) isolating sample DNA;

(b) deriving a set of target DNA fragments, said set of target fragments comprising concomitantly amplifiable target DNA fragments from the sample DNA;

(c) treating the target DNA fragments obtained in step (b) a probe restriction endonuclease reagent;

(d) amplifying the amplifiable probe restriction endonuclease reagent treated target DNA fragments of step(c); and

(e) analyzing the DNA of step (d) to determine which target fragments are amplified and/or which target fragments are not amplified; and wherein amplified target fragments lack a recognition site for the probe restriction endonuclease reagent and target fragments having a recognition site for a probe restriction endonuclease reagent are not amplified.

In one aspect, the present invention is directed to RAA-methods, which comprise the preparation of concomitantly amplifiable DNA segments by digestion of the starting DNA with one or more restriction endonucleases, collectively referred to herein as sampling enzymes. This method is herein referred to as format-I RAA and is diagrammed in Figure 2. The digested starting DNA may be further modified at its termini by the addition of adapters, which may serve to prime an amplification reaction (see Figure 2). Once sample DNA is obtained, it is treated with a different restriction enzyme, the probing enzyme also referred to as a probe restriction endonuclease reagent. A combination of probing and sampling enzymes are chosen such that a

substantial fraction of the sample fragments contain a single recognition site for the probe endonuclease reagent. In general, probe enzymes used with format-I RAA preferably have as a recognition site a nucleotide sequence of less than six nucleotides.

In another aspect, the present invention is directed to methods for format-II RAA for the detection of ESPs, as diagrammed in Figure 3. Format-II RAA operates on the same principal as format-I RAA except that the sample amplicons need not be DNA fragments, but are rather defined regions of a genome amplifiable with specific primer pairs. The amplicons of the format-II RAA are identified on the basis of sequence data; e.g. the sequence of ESP-containing restriction fragments identified using format-I RAA method or otherwise known SNPs affecting endonuclease cleavage sites. In format-II RAA, the test DNA to be analyzed is treated with a probe restriction endonuclease reagent, followed by the concomitant amplification of regions of the treated DNA (amplicons) using predetermined primers using, for example, the polymerase chain reaction as described herein. The analysis of the amplification products then proceeds as described in the format-I RAA methods described herein. As with format-I RAA, an ESP is genotyped by the presence or absence of a recognition site for the probe restriction endonuclease reagent.

In yet another aspect, the present invention is directed to methods for format-III RAA. In essence, format-III RAA consists of a combination of the format-I and format-II approaches. One of such combinations is diagrammed in Figure 4. Test DNA, digested or not with a probe endonuclease reagent, is sampled with a pair of endonuclease reagents and the resulting fragments are co- as described in the format-I assay amplified (this step is referred to as the pre-amplification step). These pre-amplification mixtures are, in turn, used as templates for a format-II type of PCR reaction in which multiple ESP-containing regions are selectively co-amplified using specific primer sets. The analysis of the amplification products then proceeds as described before. The advantages of format-III RAA are that the stepwise amplification facilitates the multiplex PCR of the ESP-specific amplicons and lowers the amount of starting material required to interrogate all the ESPs.

Arrays, or microarrays of probe DNA wherein the probe DNAs are useful in the detection of ESPs are also encompassed by the present invention.

Informative probe DNAs are prepared and identified as described in detail below and are then attached to a substrate for use in the hybridization reactions with concomitantly amplifiable DNA after treatment with a probe restriction endonuclease reagent and subsequent amplification.

Since the method of the invention is based on the detection of a particular kind of DNA polymorphism, which occurs in DNA of any organism, the invention will be universally applicable. The methods of the present invention may be used to genotype ESPs in a wide variety of organisms from prokaryotic organisms, such as bacteria, through complex eukaryotic organisms, viruses, or any organism having a genome however simple or complex. The methods may also be used for the analysis of extrachromosomal DNA, the DNA found in certain cellular organelles, cDNA preparations, or DNA libraries, such as yeast artificial chromosome libraries and others. Furthermore, based on the large body of DNA sequence data at hand, it is predicted that the genomes of higher organisms carry several hundreds of thousands of such DNA polymorphism. Consequently, the new method is capable of diagnosing the immense number of genetic markers that are needed to unravel complex traits. The method is of tremendous value for high throughput genetic analysis in the emerging field of pharmacogenomics. Similarly, the method has great potential in the field of animal and plant breeding, where high resolution genetic analysis will be needed to identify the genes involved in quantitative agronomic traits.

Various aspects of the present invention are described in more detail below (*see* Detailed Description of the Invention). Variations in each of these aspects will be readily appreciated by one of ordinary skill in the art and one with the scope of the invention.

Brief Description of the Drawings

Figure 1 depicts the general concept of the Restricted Amplicon Assay. The vertical arrows indicate the positions of the ESPs. The open circles denote the probing enzyme sites that are present, while the closed circles denote the mutated sites. The first step involves cleavage of the test DNA with the probing endonuclease. The second step involves PCR amplification of DNA segments comprising the ESPs. The

small horizontal arrows denote the PCR primers flanking the ESPs. When cleavage occurs the DNA is cut between the PCR primers, preventing the subsequent amplification of the DNA segment comprising those ESPs. Only those DNA segments that were not cleaved are amplified. The final step comprises assaying the amplicons.

Figure 2: Diagrammed representation of format-I RAA. The vertical arrows indicate the positions of the ESPs, with the open and closed circles denoting the probing enzyme sites that are respectively present and absent. Step 1 represents the sampling enzyme cleavage step. The vertical dotted arrows indicate the positions of the sampling enzyme cleavage sites. Step 2 represents the adapter ligation step. The open lines represent the adapters ligated to the ends of the sampled restriction fragments. Step 3 represents the probing enzyme cleavage step and the small horizontal arrows denote the PCR primers matching the adapter sequences. Step 4 represents the PCR amplification step in which only the sample fragments that are not cleaved by the probing enzyme are amplified. The crossed circles represent the fragments that are not amplified.

Figure 3: Diagrammed representation of format-II RAA. The vertical arrows indicate the positions of the ESPs, with the open and closed circles denoting the probing enzyme sites that are respectively present and absent. Step 1 represents the probing enzyme cleavage step. The dotted boxes denote the DNA sequences flanking the ESP sites. Step 2 represents the PCR primer design. The small horizontal arrows denote the PCR primers flanking the ESPs Step 3 represents the PCR amplification step in which only the sample fragments that are not cleaved by the probing enzyme are amplified. The crossed circles represent the fragments that are not amplified.

Figure 4: Diagrammed representation of format-III RAA. The vertical arrows indicate the positions of the ESPs, with the open and closed circles denoting the probing enzyme sites that are respectively present and absent. Step 1 represents the sampling enzyme cleavage step. The vertical dotted arrows indicate the positions of the sampling enzyme cleavage sites. Step 2 represents the pre-amplification step in which the sampled fragments are amplified. Step 3 represents the probing enzyme cleavage step. Step 4 represents the PCR primer design. The small horizontal arrows denote the PCR primers flanking the ESPs. Step 5 represents the PCR amplification step in which

only the sample fragments that are not cleaved by the probing enzyme are amplified. The crossed circles represent the fragments that are not amplified.

Figure 5: Graphic representation of target fragments produced by cleavage with a hexacutter (full arrows) and a tetracutter (dotted arrows) restriction enzyme. Two types of fragments are produced: type I fragments (dotted lines) carrying two tetracutter ends and type II fragments (full lines) carrying one hexacutter end (represented by the arrowhead) and one tetracutter end. Upon PCR amplification only the type I fragments are amplified.

Figure 6: EcoRI-BfaI fragments from ecotypes Columbia (C) and Landsberg (L) obtained after selective amplification using EcoRI and BfaI AFLP primers with respectively 2 and 3 selective nucleotides. The fragment patterns were obtained respectively without probing enzyme (no enzyme) and after digestion with the MseI probing enzyme. It is noted that most of the larger fragments do not survive after MseI digestion, while the majority of the smaller fragments survive the treatment. The differences between the ecotypes Columbia (C) and Landsberg (L) observed after MseI digestion, marked by the arrows represent ESP carrying fragments. The differences found without MseI digestion, marked by the stars represent typical AFLP polymorphisms.

Figure 7: Hybridization patterns obtained on the Arabidopsis micro-arrays. The layout of the Arabidopsis micro-array is as follows: the left panel contains the ESP fragment probes derived from Columbia (upper half) and Landsberg (lower half), while the right panel contains the control monomorphic probes with respectively the negative control fragments (-control) always carrying a probing endonuclease site and the positive control fragments (+control) carrying no probing endonuclease site. The upper part of the figure shows the hybridization patterns obtained with uncleaved sample DNA, while the lower part of the figure shows the hybridization patterns obtained with cleaved sample DNA. The dark-grey circles code is as follows: light-grey circles represent hybridization with the Cy3-labeled fragments, dark-grey circles represent hybridization with the Cy5-labeled fragments, black circles represents hybridization with both the Cy3-labeled and the Cy5-labeled fragments, and open circles represent no hybridization. In this figure of a set of idealized results is

presented. The hybridization patterns with the uncleaved sample DNA shows that all probes detect sequences in both ecotypes, while the hybridization patterns with the cleaved sample DNA show that the ESP fragment probes detect only the sequences in the respective ecotypes from which the ESP fragments were isolated. In addition, fragments carrying no site for the probing enzyme, detect sequences in both ecotypes, while fragments that always carry a site for the probing enzyme do not show a hybridization signal.

Figure 8: Hybridization patterns obtained on the corn micro-arrays. The layout of the corn micro-array is as follows: the left panel of probes contains random fragments derived from B73, while the right panel contains Mo17-fragments. The figure shows four hybridization patterns obtained with respectively uncleaved sample DNA, MseI-cleaved, Tsp509I-cleaved and AluI-cleaved cleaved sample DNA. The uncleaved sample DNA hybridization pattern shows probes that hybridize only to B73 (light-grey circles), respectively Mo17 (dark-grey circles) fragments, which represent polymorphisms resulting from mutations in the sample enzyme recognition sites. The cross in the circle indicates that these probes are eliminated from the analysis. The cleaved sample DNA hybridization patterns show that the majority of the probes do not give a hybridization signal, indicating that their cognate fragments are cleaved by the probing enzyme. Most of the probes giving a signal hybridize to both sample DNAs. Those that hybridize to only one of the sample DNAs and that were eliminated represent fragments carrying ESPs. The arrows denote the probes that were retained for further analysis.

Detailed Description of the Invention

The term "SNP" means Single Nucleotide Polymorphism, i.e. a polymorphism involving the mutation of a single base-pair.

The term "ESP" means Endonuclease Site Polymorphism, i.e. a polymorphism involving two alleles one of which is cleaved by an endonuclease reagent while the other exhibits (at least partial) resistance to cleavage by the same endonuclease under the same conditions.

The phrase "(restriction) endonuclease reagent" refers to a reagent that consists of one or more enzymes and that cleaves nucleic acids with a certain

specificity, i.e. cleavage involves recognition of a particular sequence or set of sequences in the target DNA. Endonuclease reagents include but are not limited to the common type II restriction enzymes.

The term "sampling endonuclease(s)" or "sampling enzyme(s)" refers to an endonuclease reagent used to derive sets of fragments from the sample DNA. The term "probing endonuclease(s)" or "probing enzyme(s)" refers to an endonuclease reagent used to probe the allelic state at specific ESP-sites.

The term "polymorphism" refers to the existence of two or more alleles at significant frequencies (≥1 %) in the population; polymorphism at a single chromosomal location constitutes a genetic marker.

The term "micro-satellite (DNA)" refers to a small array (often less than 0.1 kb) of tandem repeats of a very simple sequence, often 1 to 4 base-pair. Variability at such a locus is the basis of many genetic markers.

The term "mutation" means a heritable alteration in the DNA sequence.

The term "allele" refers to one of several alternative sequence variants at a specific locus.

The term "genotype" is commonly known to mean (i) the genetic constitution of an individual, or (ii) the types of allele found at a locus in an individual.

The term "haplotype" refers to the genotype at a series of linked loci on a single chromosome.

The term "sample DNA" or "sample fragments" refers to the set of fragments or amplicons derived from the starting DNA by the RAA method.

The term "zygosity" refers to the homozygous or heterozygous state.

The term "homozygosity/homozygous" refers to the presence of identical alleles at a locus.

The term "heterozygosity/heterozygous" refers to the presence of different alleles at a locus.

The term "CpG" means a dinucleotide with a cytosine at the 5'-side and a guanine at the 3'-side. CpG is relatively rare in mammalian DNA because of the tendency for the cytosine to be methylated and subsequently mutate to thymine by deamination.

The term "ecotype" refers to a naturally occurring (plant) variety; race.

The term "bi-allelic" refers to a polymorphic locus characterized by two different alleles.

The terms "microarray" and "(DNA-)chip" refer to a multitude of spatially addressable nucleic acids that serve as probes. The microarray may be used in the form of a planar solid support, a bead, a sphere, or a polyhedron. Fabrication is done either by in situ combinatorial synthesis of oligonucleotides using photolithography, or by robotic spotting of off-chip prepared DNA onto a solid surface.

The methods of the present invention differs conceptually from previously described restriction enzyme-dependent assays (*supra*) that essentially detect a fragment length polymorphism. With the present method, starting DNA is restricted prior to the amplification reaction and, rather than analyzing the obtained amplification product, the presence or absence of amplification is measured to determine the allelic state at an ESP site. The treated DNA is preferably amplified by using a polymerase chain reaction and is preferably analyzed by means of hybridization against arrays of probe DNAs. With the present method, a sample-amplicon, and consequently a hybridization signal, is either present or virtually absent. This feature represents a major advantage in that it results in a more accurate distinction between variable nucleotides than is possible by differential hybridization to allele-specific oligonucleotides, and because it greatly facilitates the identification of a set of generally useful hybridization conditions. Also, the methods of the invention permit the use of both oligonucleotides as well as DNA fragments as probe DNAs. While hybridization to arrays allows the simultaneous analysis of a large number of ESPs, it should be clear that the amplification of sample DNA, treated with probe restriction endonuclease reagent, can be analyzed by any of a variety of methods well known in the art. In these methods, an ESP is identified either by the presence of a recognition site for the probe restriction endonuclease reagent (which will result in the failure of the sample DNA to amplify) or by the loss of a recognition site which will allow amplification of an otherwise unamplifiable sample DNA. Alternative methods include, but are not limited to, gel-electrophoretic analysis, and the TaqMan assay [Holland P. M. *et al., Proc.*

Natl. Acad. Sci. **88**: 7276-7280 (1991); with the latter assay detection is done during rather than after the amplification reaction].

One of the advantages of the method of the invention is the ability to calibrate the measured signal against that obtained in a control experiment where digestion with the probe restriction endonuclease reagent is omitted. Comparison of the respective hybridization signals, following various corrections and normalization procedures, is essential for the genotyping of ESPs and the accurate determination of the zygosity. The cleaved and uncleaved material can, in principle, be hybridized separately but a preferred method consists of hybridizing a mixture of the differentially labeled samples to the same array. The present invention is exemplified by several specific formats described below.

(I) Format-I RAA: Choice of sampling and probing restriction endonuclease reagents. In one of its embodiments the present invention is directed to methods for detecting ESPs in a "restricted amplicon assay" (RAA) which comprises preparing concomitantly amplifiable restriction fragments from the starting DNA (sample DNA). When generating discrete sets of DNA fragments from genomic DNA, the following parameters are important: the average fragment size and the total number of fragments. The optimal fragment size for use in the methods (and materials) of the present invention is a trade off; the fragments must be sufficiently small for amplification with roughly equal efficiency (in general ≤500 base pairs) and large enough for having on average one cleavage site for the probing endonuclease reagent. In addition to average fragment size, the number of fragments determine the complexity of the sample DNA which is critical in view of the limitations of the detection sensitivity of micro-array hybridization. In general, the current state of the art of microarray hybridization is such that the number of sample fragments should not exceed 100,000. All of the above-mentioned requisites can be met by the appropriate choice of sampling and probing enzymes. A preferred method of the present invention to prepare sample DNAs (amplicons) involves the use of two different sampling enzymes, a rare cutter endonuclease (*e.g.*, hexacutter) combined with a frequent cutter endonuclease (*e.g.*, tetracutter), as described in EP 0 534 858 A1 which describes a method called AFLP and which is incorporated herein by reference. As can be seen

from Figure 5, the rare cutter enzyme produces large fragments that upon cleavage with the frequent cutter enzyme are cut into a number of smaller fragments. This dual cleavage generates two types of fragments: the majority having both ends produced by the frequent cutter (type I) and a minority of fragments having a rare cutter end and a frequent cutter end (type II). After ligating different adapters to each of the ends and using appropriate primers targeted to the ends of the fragments, only the type II fragments will be amplified efficiently (see Figure 5). The type I fragments amplify with greatly reduced efficiency presumably because the synthetic sequences at the two ends constitute an inverted repeat. In general the type II fragments will amplify synchronously using a single PCR primer pair that attaches to the ends of the fragments. The size limit is typically around 500 base pairs, but can be increased by using a different DNA polymerase and other reaction conditions. Thus, as outlined above the number of amplifiable fragments will be determined primarily by the choice of the rare cutter restriction enzyme. By approximation, this number equals two times the number of cleavage sites for the rare cutter. In a preferred embodiment, restriction enzymes recognizing 6 nucleotides (hexacutters) or more are used as rare cutters. The use of a frequent cutter recognizing 4 nucleotides (tetracutter) as second sampling enzyme results in the production of fragments in the optimal size range for co-amplification. As probe restriction endonuclease reagents, different tetracutter or pentacutter enzymes can be used. The probe restriction endonuclease reagent and the frequent cutter sampling enzyme should preferably be chosen such that the ratio of the cleavage frequencies of probing over sampling reagent is >0.5 and <3. This will ensure that a substantial fraction of the target fragments are cleaved once by the probing enzyme. It is noted that ESPs cannot be genotyped when the fragments are cleaved more than once by the probing enzyme. Also, it should be recognized that cleavage with the probe restriction endonuclease reagent results in a significant reduction (typically 2-4 fold) of the fragment complexity.

Alternative schemes - different from the one described above - that meet the requisites of sample complexity, average fragment size, and occurrence frequency of the probe reagent and that will perform equally well, will be readily apparent to one of ordinary skill in the art. Alternative schemes may include the use of pairs of

frequent cutters, followed by selective amplification (described in EP 0 534 858 A1), or the use of type IIS restriction enzymes. Type IIS restriction enzymes are characterized by an asymmetric recognition sequence. Most of these enzymes cleave at a defined distance to one side of the recognition site and generate single stranded overhangs that have different sequences. Ligation of adaptor sequences that are complementary to only one type of overhang allows the amplification of specific subsets of fragments [Kikuya Kato, *Nucleic Acids Res.* 23: 3685-3690 (1995)]. With this strategy the set of fragments obtained with the sampling enzymes can be broken up in a defined number of complementary and roughly equally complex subsets. Thus, with these enzymes it is possible to tune the complexity of the sample. The same strategy can be applied by making use of type II enzymes that have an interrupted palindromic recognition sequence.

Type of mutations detected by format-I RAA: In essence the method of the invention aims to detect mutations affecting the recognition sequences of the site-specific probe endonuclease reagents. When the probe enzyme cleaves a sample fragment, it is prevented from being amplified and as a consequence the fragment will not give a hybridization signal with its cognate probe. Mutations affecting the recognition sequence of the probe enzyme will allow amplification of the sample fragment and will restore the hybridization signal. It is recognized that mutations other than those affecting the probe enzyme recognition sites may affect the hybridization signals. In particular, mutations affecting the recognition sites of the sampling enzymes may also lead to a loss of hybridization signal. Consequently, the mere detection of a hybridization difference between two samples does not qualify the difference as being due to an ESP for the probing enzyme. For this one must also assay the two samples without probing enzyme cleavage; only those differences that are correlated with the cleavage by the probing enzyme qualify as genuine ESPs as defined according to the present invention. Therefore, a preferred embodiment of the methods of the present invention comprise the comparison of the hybridization signals obtained with and without cleavage of the same starting material by the probe endonuclease reagent. Preferably, the digested and undigested sample DNAs are differentially

labeled such that equivalent amounts of the material can be mixed and hybridized against the same array of probes. It is noted that a further advantage of measuring the relative hybridization signals obtained with digested and undigested sample DNAs, is that the signal given by the undigested sample DNA serves as an internal control for correcting variations in amplification and hybridization.

Identification and design of informative probes to detect ESP-harboring fragments. In a preferred embodiment of the present invention sample DNAs (amplicons) are hybridized to micro-arrays comprising a set of probe DNAs which are designed such that each probe will hybridize specifically to one sample DNA fragment. For each set of sample DNA fragments a specific set of probes are developed that will detect all the ESPs present in the set of sample DNAs. Since in most applications only a (minor) fraction of the sample DNAs will actually carry an ESP for a particular probing reagent, the set of probe DNAs will preferably consist of a subset of the sample DNA fragments that are informative in that they hybridize to ESP-harboring sample fragments. Preferably, the probes are highly specific for the ESP-carrying sample fragments, and do not cross-hybridize with other fragments in the sample. This feature is verified by testing the candidate probes in control hybridization assays. When developing or designing the probes care should be taken to avoid hybridization of the labeled primer used to amplify the sample fragments. When the probes correspond to a subset of the sample fragments, preferably an alternative set of adaptors should be used for their amplification.

The sections below describe different approaches that may be used to assemble sets of unique probe DNAs for fabricating the micro-arrays. Three alternative approaches are presented, and their choice is determined primarily by the degree of nucleotide sequence variation, and hence the ESP frequency, present in the species under study.

(1) Direct screening. When the ESP frequency is high, such that 10% or more of the sample fragments carry ESPs, a realistic approach for assembling ESP probes is to array individual sample fragments and test which of them detect an ESP in the test material under study. The advantage of this approach is that the same set of

fragments can be tested with different probe enzymes. After the screening one will retain only those probes that yield a clear-cut difference in hybridization between the different test DNAs. This approach is illustrated in Example 2.

(2) Gel-based screening. With genomic DNA exhibiting intermediate ESP frequencies (a few %), useful probes can be identified with a gel-based screening approach in which the ESPs are identified by comparing the patterns of sample fragments obtained from cleaved and uncleaved genomic DNA of various individuals. The polymorphic fragments can then be isolated from the gel and cloned or amplified. In a second phase, these probe-fragments are verified in a micro-array hybridization assay. This approach is illustrated in Example 1.

(3) Batch-wise hybridization selection method. Since both approaches described above are inefficient and labor intensive when the ESP frequency is low (<1%), it is advantageous to directly select or enrich ESP-carrying fragments. Such an approach is described in greater detail in Example 3.

The methods of the invention can be used with any type of micro-array: spotted ESP-carrying fragments, spotted oligonucleotides or oligonucleotides synthesized on solid supports using photolithography [Fodor S. P. A. *et al., Science* **251**: 767-773 (1991)]. Oligonucleotide probes can easily be designed based on the nucleotide sequences of the ESP-carrying fragments. Also, the methods of the invention are not limited to the use of planar arrays containing spatially addressable probes. A person of skill in the artwill recognize that the methods may alos employ a multitude of identifiable solid phase particles (e.g. beads, spheres, and polyhedron), each carrying a different probe. Examples of such use are described by Fulton, R. [U.S. Patent No. 5,736,330] and Mandecki, W. [U.S. Patent No. 5,736,332].

(II) Format-II RAA General outline

The 'format-I RAA' - as described above - can be converted to a 'format-II assay' when sufficient sequence information of ESP-containing sample fragments becomes known. Format-II RAAs can also be designed on the basis of the known sequences of genomic regions that harbor an ESP and that are available through publicly accessible databases. The approach involves the targeted sampling of starting

material and consists of the design of dedicated primer pairs that flank the ESP sites. Like in format-I RAA, if the site is intact, the starting DNA will be cleaved and no PCR product will be generated. Only when the site is mutated will the amplicon be generated. In practice, multiple ESP-containing genomic regions are co-amplified after cleavage with the probing restriction endonuclease reagent. The ultimate sample DNA used in the hybridization reaction is composed of several such multiplex PCR reactions pooled together. The feasibility of this approach is evidenced by the recent paper of Wang *et al.*, *Science* **280**: 1077-1082 (1998), incorporated herein by reference. The methods for format-II RAA described here are identical to the approach described by Wang *et al.*, in the way certain allelic regions are co-amplified, but fundamentally different in the way they are diagnosed. The present method takes advantage of the clear distinction between having or not having an amplicon depending upon the allelic state of the endonuclease target site. The Wang *et al.* approach in contrast relies on the detection of a hybridization difference as a result of a single nucleotide variation in the PCR product. This requires a much more elaborate and redundant hybridization assay.

Similar to format-I RAA, a preferred method consists of comparing the hybridization signals obtained with and without cleavage with the probe restriction endonuclease reagent. Preferably, the respective amplification reactions are differentially labeled such that the resulting amplicons can be mixed and hybridized against the same array of probes.

Preferred methods of the format-II RAA are those wherein - of each PCR primer pair - that primer that remained unlabeled is used as hybridization probe for the corresponding amplicon. This ensures that the excess unincorporated labeled primer as well as the primer extension products obtained with this primer cannot anneal to the arrayed probe. Also, the unlabeled PCR primer is complementary to the labeled strand of the amplicon.

Furthermore, the format-II RAA method provides a means to monitor mutations in specific genes or loci in addition to scanning the entire genome. Indeed, sets of PCR primers that target ESPs in a specific gene or chromosome region can be assembled.

An RAA assay with positive detection of both alleles:. It is recognized that the 'present/absent-score' of the RAA assay cannot (always) distinguish between different mutations that can affect cleavage by the probe restriction endonuclease reagent. In practice, an ESP should not be assayed when available evidence indicates the existence of two or more such mutations at significant frequencies in the population.

In a preferred embodiment the present invention is directed to the detection of SNPs that result in the simultaneous loss and gain of a restriction enzyme recognition site, *i.e.* both alleles are associated with a different recognition site. HgaI (GACGC) and SfaNI (GATGC) are an example of such reciprocal sites. Use of both probing endonuclease reagents in side-by-side experiments excludes alternative alleles and results in easy determination of the zygosity (refer to Example 4).

Multi-allelic haplotyping:. A single ESP represents a bi-allelic marker, which is less informative than a variable micro-satellite, which has multiple alleles. It is possible however to compensate for the lower information content by identifying several ESPs on a specific chromosomal region. Format-II RAA lends itself readily to such an approach and involves the design of a primer pair that encompasses a region with a single site for the various selected probe endonuclease reagents. It should be recognized as one of the advantages of the present method that multiple ESPs on a sample amplicon can be interrogated with a single probe. Furthermore, use of the probing enzymes, either separately or in various combinations, in parallel experiments allows the construction of the haplotypes for the ESPs under study. In general, the statistical associations between traits and specific chromosome regions may be more apparent when haplotypes rather than individual markers are used.

(III) Format-III RAA:

In a general sense, the format-III RAA represents a method of choice for very high-density SNP genotyping because it provides a means to overcome the intrinsic limitations of both the format-I RAA and the format-II RAA. This is essentially achieved by performing a stepwise amplification involving a pre-

amplification of sample fragments followed by amplification using multiplexed specific primers. The principal advantage of the pre-amplification step is to reduce the complexity of the starting DNA, and thus to provide a more favorable starting point for performing multiplex PCR reactions. It is noted that this improvement is generally applicable to any multiplex PCR reaction, and is not limited to the methods of the present invention. Such an approach can also be used when for example SNPs are genotyped using the methods described by Wang *et al.*

The principal limitation of the format-I RAA lies in the complexity of the sample DNA that is hybridized to the microarray. Because the second round of amplification in format-III yields only very small amplicons, which are all informative, there is no longer a limitation in number of sample fragments that are interrogated. In fact the entire genome may be sampled in a series of parallel pre-amplification reactions and the amplicons generated in the different multiplex PCR reaction can then be pooled together and hybridized to the microarray.

Likewise, the format-III RAA represents preferred methods of format-II RAA, especially when the ESPs under study are located on fragments generated by one set of sampling endonuclease reagents. Such stepwise amplification comprises the co-amplification of sample fragments with a single pair of primers, followed by the selective amplification of sets of specific ESP-containing regions (see Figure 5). The principal advantage of the format-III RAA over format-II RAA is that the initial amplification of the sampling fragments - representing only a fraction of the total genome - lowers the amount of starting material required to interrogate a very large numbers of ESPs. Also, the approach will facilitate the multiplex amplification of the ESP-specific amplicons and, consequently, yield a more robust assay.

One preferred embodiment of the format-III RAA is its use to genotype large numbers of ESPs identified through the use of the format-I RAA. Indeed, format-I RAA offers a rapid means to discover large numbers of ESPs in any biological species where no large body of sequence information is or will be available. Format-I RAA enables one to discover many sets of ESPs for a number of different probing enzymes. Using the format-I RAA, each set of ESPs must be assayed on a different microarray, because otherwise signals for the same sample fragment will overlap with

one another, and thus preclude the proper ESP genotype to be determined. Using the format-III RAA, the ESPs identified with different probing enzymes are now assayed together on one single microarray, without overlap between the different ESPs. The reason is that the overlap in the format-I RAA is caused by the non-informative sample fragments that are always co-amplified with the ESP fragments. These are eliminated from the mixture by the specific PCR amplification. This embodiment is illustrated in Examples 2 and 3.

Another preferred embodiment of the format-III RAA is its use to genotype large numbers of SNPs identified in high-throughput sequencing of genomic DNA from different individuals from a given species. Given the generally recognized importance of SNPs for the development of high-resolution genotyping methods, sequenced SNPs can be expected to accumulate in large numbers in publicly available databases in the near future. In particular, in the field of human genetic analysis, SNPs will be discovered at a rapidly increasing rate through the massive genome sequencing programs now in progress. A similar evolution may be anticipated for many other species. Hence we decided to perform an *in silico* analysis of known human SNPs to further investigate the potential of the invention. More particularly we have analyzed the 3,358 SNP sequences present in the SNP database of the Whitehead Institute [Wang *et al., Science* **280**: 1077-1082 (1998)]. We have determined how many of these SNPs represent an ESP for each of 34 known palindromic and non-palindromic tetra- and penta-nucleotide restriction recognition sequences. When extrapolating this number to the total number of ESPs in the human genome - assuming a grand total of 3 million ESPs - it appears that the number of detectable ESPs per probing restriction enzyme is in the range of 25.000 to 150.000. A cumulative analysis reveals that 53% of the SNPs affect at least one of the 34 restriction sites; a total of 28% affect the recognition site for one of the available tetracutter enzymes. The principal conclusion from this analysis is that many of the considered enzymes - used as probing enzymes according to the methods of the present invention - will interrogate sufficient SNPs to be able to built a high-density map of the human genome. It should also be noted that the use of multiple probing enzymes is easily accommodated in the targeted assay because the sample has to be subdivided anyway over a number f parallel multiplex PCR

reactions. This embodiment is illustrated in Example 4.

It is noted that the format-III RAA may be performed according to different procedures. One such procedure is diagrammed in Figure 5, in which the test DNA is first sampled using a sampling endonuclease reagent, pre-amplified and then treated with the probing endonuclease reagent. Variations on this procedure are readily recognized by those skilled in the art and include for example, concomitant treatment of the test DNA with both the sampling and the probing endonuclease reagents and the preparation of sampled DNA fragments using arbitrary PCR priming methods [Williams *et al., Nucleic Acids Res.* **18**: 6531-6535 (1990)]. Note that in case the treatment with the probing endonuclease reagent is performed prior to the pre-amplification, the subsequent amplification can be performed with any pair of PCR primers directed against the ESP carrying fragments, and thus overcoming the limitation of using PCR primers flanking the ESPs.

Table I. Analysis of 3,358 SNPs in the Whitehead SNP database. The table lists the number of SNPs that represent an ESP for various probing enzymes. The last column shows the estimated number of ESPs for each enzyme in the entire human genome (refer to text for details).

Type of probing reagent	enzyme	site	ESPs	total number of ESPs
Tetracutter enzymes	Tsp509I	AATT	122	109.000
	MaeII	ACGT	106	95.000
	AluI	AGCT	98	88.000
	NlaIII	CATG	158	141.000
	MspI	CCGG	77	69.000
	BstUI	CGCG	27	24.000
	BfaI	CTAG	67	60.000
	Sau3A	GATC	58	52.000
	HinPI	GCGC	49	44.000
	HaeIII	GGCC	52	46.000
	Csp6I	GTAC	71	63.000
	TaqI	TCGA	50	45.000
	MseI	TTAA	109	97.000
Pentacutter enzymes	Tsp4CI	AGNCT	114	102.000
	BssKI	CCNGG	79	71.000
	DdeI	CTNAG	122	109.000
	HinfI	GANTC	77	69.000
	Fnu4HI	GCNGC	71	63.000
	Sau96I	GGNCC	64	57.000
	MaeIII	GTNAC	70	63.000
Non-palindromic enzymes	AciI	CCGC	111	99.000
	MnlI	CCTC	175	156.000
	BbvI	GCAGC	65	58.000
	BsmAI	GTCTC	67	60.000
	BsmFI	GGGAC	39	35.000
	FokI	GGATG	66	59.000
	HgaI	GACGC	31	28.000
	PleI	GAGTC	39	35.000
	SfaNI	GCATC	51	46.000
	AlwI	GGATC	37	33.000
	BsrI	ACTGG	76	68.000
	HphI	GGTGA	69	62.000
	MboII	GAAGA	85	76.000
	TspRI	CAGTG	94	84.000

The following illustrative examples were chosen to represent the spectrum of genomic complexities and the spectrum of degrees of genetic variation which are susceptible to analysis using the methods of the present invention:

Example 1 describes analysis of Arabidopsis (low genomic complexity, low genetic variation).

Example 2 describes genetic analysis of corn (high genomic complexity, high genetic variation).

Examples 3 and 4 describe genetic analysis in humans (high genomic complexity, low genetic variation).

Numbers given in the examples, and that relate to the occurrence frequency of certain restriction sites as well as the average size of the generated fragments are in part based on computer simulations using publicly available DNA sequences.

Example 1

Genetic Analysis in Arabidopsis

In this example, a fragment analysis-based approach is used to generate a set of genomic fragments carrying ESPs between the Arabidopsis ecotypes Landsberg and Columbia, which are commonly used for genetic studies in the model organism. Arabidopsis is an example of a low complexity genome (size ~120 Mb), and the two ecotypes exhibit a moderate level of genetic variability. Previous studies have revealed that the average nucleotide sequence variation between the two ecotypes is in the order 1 polymorphism in 150 nucleotides. Consequently, the fraction of fragments expected to carry an ESP for tetranucleotide recognizing restriction enzymes is expected to be in the range of 2.5% (1:40). With such a low frequency, it is helpful to use a selection procedure to isolate the rare fragments containing ESPs.

In essence the procedure described in this example comprises the following steps:

4) Identification of a set of about 200 genomic fragments carrying Landsberg/Columbia ESPs using a gel-electrophoretic approach.
5) Isolation and characterization of the ESP carrying DNA fragments (ESP fragments).
6) Generation of micro-arrays with the ESP fragments
7) Confirmation of the ESPs by hybridization.

Step 1. Identification of ESP fragments.

Sampling enzymes. In the present example EcoRI, a restriction enzyme recognizing 6 nucleotides (hexacutter), in combination with BfaI, a restriction enzyme recognizing 4 nucleotides (tetracutter), are chosen as sampling enzymes. From the random frequency of occurrence of 6 nucleotide sequences (every 4,000 bases), the number of sites for hexacutter restriction enzymes in this genome is predicted to be in the range of 30,000. In addition to cleavage with a hexacutter, the genomic DNA is also cut with a tetracutter so as to generate PCR amplifiable fragments of an average size of a few hundred base pairs. Cleavage with the two enzymes gives rise to two types of fragments: a majority of fragments resulting from cleavage by the tetracutter

enzyme alone and a smaller set of fragments produced by the two enzymes (see Figure 5). Since the majority of the hexacutter fragments will give rise to two fragments having a hexacutter end and a tetracutter end (*see* Figure 5), this procedure will yield a mixture of about 60,000 fragments of this type. Upon amplification using the procedure described below only the fragments carrying a tetracutter end and a hexacutter end are amplified efficiently (Figure 5).

Probing enzymes. As probing enzymes many different tetracutter enzymes can be used. Ideally, the probing enzyme cleaves most of the sample fragments once. Because plant DNA has a high AT content, the preferred tetracutters are those that have an AT bias in their recognition sequence. In general, the choice of an optimal tetracutter may be determined by particular features of the genome being analyzed (*e.g.*, AT and GC content). In the present example, MseI (recognition site = TTAA) was chosen. Tsp509I (recognition site = AATT) is an alternative. It is also conceivable to use mixtures of two or more tetracutter enzymes. The EcoRI-BfaI sample/target fragments that are cleaved and not cleaved with the MseI probing enzyme are referred to as cleaved and uncleaved sample/target DNA, respectively.

Screening for ESP carrying fragments. To detect ESP fragments, subsets of uncleaved and cleaved EcoRI-BfaI sample fragments from both ecotypes are amplified and the amplicons are compared following gel-electrophoretic fractionation. Subsets of the EcoRI-BfaI sample fragments are selectively amplified as described [Vos, P. *et al.*, *Nucleic Acids Res.* **23**: 4407-4414 (1995); Zabeau, M. and Vos, P., European Patent Application EP 0534858 (1993) both of which are incorporated herein by reference]. Given the complexity of the sample (~50,000 fragments), the selective amplifications are performed with EcoRI and BfaI primers having two and three selective nucleotides, respectively. This equals 1024 (16 x 64) different selective amplification reactions.

The experimental procedure described by Vos P. *et al.* is followed except that the template fragments are incubated at 65°C during 10 minutes to heat-inactivate the T4 ligase enzyme, and, when applicable, digested with the probing enzyme prior to amplification. The structures of the EcoRI and BfaI adaptors are as follows [*see, e.g.*, Vos, P. *et al.*, ***supra***]:

5'-CTCGTAGACTGCGTACC (SEQ ID NO: 1)
CATCTGACGCATGGTTAA-5' (SEQ ID NO: 2)

5'-GACGATGAGTCCTGAG (SEQ ID NO: 3)
TACTCAGGACTCAT-5' (SEQ ID NO: 4)

The EcoRI (radiolabeled by 5'-phosphorylation) and BfaI primers, having two and three selective nucleotides, respectively, have the following sequences (where N represents A, C, G, or T):

5'-GACTGCGTACCAATTCNN (SEQ ID NO: 5)

5'-GATGAGTCCTGAGTAGNNN (SEQ ID NO: 6)

Using these reagents, most of the obtainable target fragments contain a cleavage site for the probing enzyme and, consequently, will not be amplified when the target DNA is cleaved. Most of the fragments that survive the treatment with the probing enzyme occur in both ecotypes, and thus carry no ESP. Occasionally fragments are found that appear in both ecotypes when the target DNA is not digested and that are present in only one of the two ecotypes after digestion. These represent true ESPs for the probing enzyme. In addition, fragments will also be found that show typical AFLP-polymorphism between the two ecotypes [Vos, P. *et al.*, *Nucleic Acids Res.* **23**: 4407-4414 (1995)]. Such polymorphisms are apparent in the fragment patterns obtainable with the undigested sample DNAs. A typical result is shown in Figure 6 in which the electrophoretic patterns are shown of selectively amplified EcoRI-BfaI fragments from the Ecotypes Columbia and Landsberg obtained without and with digestion with the MseI probing enzyme.

Systematic comparison of the patterns of ecotypes Columbia and Landsberg before and after digestion, allows the identification of EcoRI-BfaI sample amplicons that carry an ESP for the probing enzyme. Using MseI as probing enzyme, it is estimated that a total of ~200 polymorphic fragments which are present in only one of the ecotypes can be identified.

Step 2. Isolation and characterization of ESP fragments.

Each of the ESP polymorphic fragments is eluted from the gel-matrix, re-amplified and cloned into a suitable plasmid vector (e.g. TA cloning system; Invitrogen, Carlsbad, CA, U.S.A.). In each case, two clones are selected for sequence determination. Most duplicate clones will yield the same sequence. Duplicate clones that gave different sequences were not retained for further work. Since the nucleotide sequence of over one third of the Arabidopsis genome is available in the public databases (*e.g.*, Genbank), the chromosomal location of one third of the ESP fragments can be determined by matching the fragment sequences to the genomic sequence. Furthermore since the genomic sequence is derived from ecotype Columbia, we expect a perfect match with the fragment sequences isolated from the same ecotype. The sequences of the fragments isolated from ecotype Landsberg will reveal single nucleotide differences, amongst which the potential restriction site mutations, affecting the MseI recognition sites, should be apparent.

In addition to the ESP polymorphic fragments, a number of non-polymorphic control fragments are processed in the same way. Two types of such control monomorphic fragments are isolated: fragments that do not carry a site for the probing enzyme and fragments that carry a site for the probing enzyme in both ecotypes. These fragments will serve the purpose of verifying the hybridization on the micro-arrays.

Step 3. Fabrication of ESP micro-arrays.

Micro-arrays of amplified fragments. The insert DNAs from the sequence verified clones are amplified, e.g. with the use of non-selective EcoRI and BfaI primers. PCR products are verified by agarose gel electrophoresis and retained if a single product of the correct mobility was present. Following ethanol precipitation, the resuspended PCR products are arrayed at high density on standard glass slides (25 x 76 mm) using either the Multigrid robotic spotter (GeneMachines™, Genomic Instrumentation Services Inc., Menlo Park, CA, U.S.A.) or the BioChip Arrayer™ (Packard Instrument Company, Meriden, CT, U.S.A.). The DNAs are spotted in a logical order with respect to the ecotype from which the fragments were isolated (upper

and lower panel) as shown in Figure 7. In addition, a set of DNAs from monomorphic control fragments was spotted next to the ESP fragment DNAs (right panel in Figure 7).

Micro-arrays of oligonucleotides. Based on the nucleotide sequences of the ESP fragments, oligonucleotides can be designed that can serve as hybridization probes to specifically detect each amplified sample fragment. The oligonucleotide probe should preferably match with a sequence that is located to one side of the ESP, opposite the side where the sequence targeted by the labeled primer is located. In this way the background is minimized because the linear amplification products generated by the labeled primer following digestion with the probing enzyme are not detected. The ESP fragment specific oligonucleotides are spotted in a micro-array format in exactly the same way as the amplified ESP fragments.

Step 4. Micro-array-based detection of ESPs.

Preparation of the sample DNAs. For each ecotype, sample DNA is prepared in two different ways. Genomic DNA, digested with the sampling restriction enzymes EcoRI and BfaI, was amplified either as such or after cleavage with the probing enzyme MseI. The amplification reactions are performed with a fluorescently labeled EcoRI primer and an unlabeled BfaI primer, both without selective nucleotides. The EcoRI primer is labeled by incorporation of Cy3(green)- and Cy5(red)-amidites during primer synthesis (Amersham Pharmacia Biotech, Uppsala, Sweden). For both Columbia and Landsberg, the cleaved sample was amplified with a Cy3-primer while the uncleaved fragments were amplified with a Cy5-labeled EcoRI primer. In addition, the Landsberg digested material was also amplified with a Cy5-labeled EcoRI PCR primer. Three different hybridization solutions are then prepared by mixing equal amounts (i.e. equal volumes) of the Cy3- and Cy5-labeled amplification reactions: one from the Columbia cleaved and uncleaved samples, a second from the Landsberg cleaved and uncleaved samples, and a third by mixing the differentially labeled cleaved samples of both ecotypes.

In case arrays of PCR products, rather than oligonucleotides, are used

as probes (refer to step 3), the co-amplification of the EcoRI-BfaI sample fragments is preferably accomplished with a pair of adaptors that differs from those attached to the arrayed probes. The alternative EcoRI and BfaI adaptors have the following structure:

5'-GAGCATCTGACGCATCC (SEQ ID NO: 26)
GTAGACTGCGTAGGTTAA-5' (SEQ ID NO: 27)

5'-CTGCTACTCAGGACTG (SEQ ID NO: 13)
ATGAGTCCTGACAT-5' (SEQ ID NO: 14)

The cognate non-selective EcoRI and BfaI primers have the following sequences:

5'-CTGACGCATCCAATTC (SEQ ID NO: 28)

5'-CTACTCAGGACTGTAG (SEQ ID NO: 16)

Micro-array hybridization. Each of the hybridization solutions is allowed to hybridize to the arrayed probes using protocols well known in the art. The experimental conditions depend primarily on the nature of the probes, PCR-amplified fragments versus oligonucleotides. Both types of experiments are amply described in literature: Wodicka, L. *et al.*, *Nature Biotechnol.* **15**: 1359-1367 (1997); Lockhart, D. J. *et al.*, *Nature Biotechnol.* **14**: 1675-1680 (1996); DeRisi, J. L. *et al.*, *Science* **278**: 680-686 (1997); Shalon, D. *et al.*, *Genome Res.* **6**: 639-645 (1996); Piétu, G. *et al.*, *Genome Res.* **6**: 492-503 (1196); Chee, M. *et al.*, *Science* **274**: 610-614 (1996); Wang D.G. *et al.*, *Science* **280**: 1077-1082 (1998); Winzeler E. A. *et al.*, *Science* **281**: 1194-1197 (1998), all of which are incorporated herein by reference.

A laser scanning system (ScanArray 3000; General Scanning Inc., Watertown, MA, U.S.A.) is used to detect the two-color fluorescence hybridization signals from the micro-arrays at a resolution of 10 micron per pixel. A separate scan is carried out for each of the two fluorophores used. Scanning parameters and laser power settings are adjusted to normalize the signal in the two channels (channel-1/Cy3; channel-2/Cy5). The obtained digital images were analyzed using the ImaGene™ image

analysis software (BioDiscovery Inc., Los Angeles, CA, U.S.A.). The extracted quantitative data are transferred to a spreadsheet for further analysis.

The present hybridization experiment is essentially set up as a confirmation of the gel-electrophoretic data (refer to step 1), and has, therefore, a predictable outcome. In addition, a number of control probes are included on the biochip that detect monomorphic EcoRI-BfaI Arabidopsis fragments (i.e., fragments on which a site for the probing enzyme is either present or absent in both ecotypes). The results from these control probes allow correction for background and optical cross-talk between the two channels, as well as calibration of the red and green hybridization signals. It is anticipated that the vast majority of the processed data are unambiguous with respect to the allelic state of a sample fragment and in agreement with the gel-electrophoretic analysis. Figure 7 shows a false-color representation of the idealized results of the present experiment using a fictitious array of probes. It cannot be excluded that certain hybridization results are not in agreement with the gel-electrophoretic assay and/or that certain probes do not allow unambiguous determination of the allelic state of the cognate sample fragment. Such probes should be excluded from the micro-arrays that are used to genotype experimental Arabidopsis samples, other than the Columbia and Landsberg controls used in the present illustrative example.

In routine genotyping experiments, either one of the hybridization schemes outlined above can be used. Determination of the allelic state can be done by comparing the hybridization signals obtained with and without cleavage of the starting DNA with the probe reagent. Alternatively, allele-calling could be based on a comparison of the signals obtained with the test-sample and an appropriate control (e.g. Columbia or Landsberg DNA), both cleaved with the probe endonuclease reagent. The samples that need to be compared can, in principle, be hybridized separately but a preferred method consists of hybridizing a mixture of differentially labeled samples to the same array.

Example 2

Genetic Analysis in Corn

In this example, the utility of the method of the invention for marker assisted selection applications in plant and animal breeding is illustrated. Corn has been chosen because it is a typical representative of crop species having a complex genome. The large size of the genome (2,400 Mb), the frequent occurrence of repetitive DNA sequences and the high degree of genetic variation, all constitute technical challenges. In this example, an approach based on the generation of a set of genomic fragments carrying ESPs from two well-known inbred lines of corn, B73 and Mo17 from which many of the corn elite lines are derived is used. Another reason for choosing these lines is that a well-studied recombinant inbred population derived from these lines is available. This population can be used to map the set of ESPs. The genetic map of ESP markers will prove to be an effective tool for genetic selection in corn breeding. It is evident, however, that a broader survey of the corn germplasm with a total of 10 to 20 lines will give a large number of additional ESPs (possibly 2 or 3 times as many) and will eventually result in a higher-resolution genetic map.

The ESP-harboring fragments could very well be identified by the gel-electrophoretic approach described for Arabidopsis (Example 1). However, an alternative strategy may be used given that the corn germplasm, like many crop species, exhibits a high degree of genetic variation. Indeed, based on previous studies, the average nucleotide sequence variation in the corn germplasm is estimated to be in the order of 1 difference in 15 to 30 nucleotides. This corresponds to a frequency in ESPs in the recognition sites of tetracutter restriction enzymes of 1 in 4. At this frequency it becomes feasible to directly examine arrays of random B73/Mo17-fragments for the presence of ESPs using the present RAA method without prior screening or selection. The strategy also lends itself readily to screening with several different probing enzymes.

In the present example, two different approaches for assaying ESPs are used. The first method (format-I RAA) is similar to the one described in Example 1, and detects ESPs in fragments sampled with a pair of restriction enzymes. In the second method (format-III RAA) individual ESPs are selectively amplified from the

sampled fragments with dedicated primer sets. The principal advantage of the latter approach is that ESPs detected with several different probing enzymes can be assayed simultaneously, and that multiplex amplification of ESP-specific PCR products is made considerably more robust.

In essence the procedure described in this example comprises the following steps:

8. Identification of a set of candidate ESP fragments from the inbred lines B73 and Mo17
9. Development of a corn ESP micro-array
10. Genetic mapping of a B73/Mo17 recombinant inbred population and of segregating populations

Step 1. Identification of candidate ESP fragments

Cloning of a set of sample fragments. To clone a set of random fragments from the inbred lines B73 and Mo17, the enzyme combination PstI and BfaI is used. The hexanucleotide-recognizing enzyme PstI was chosen because of the large size of the corn genome. It is estimated that this enzyme has around 30,000 sites in the corn genome. The second tetracutter-enzyme, BfaI, is expected to cleave in the majority of the cases on both sides of the PstI sites. The double digestion will therefore generate about 60,000 sample fragments with an average size of 400-500 base pairs.

Following double digestion of the genomic DNA, PstI- and BfaI-adaptors were ligated to the fragment ends and the material amplified with non-selective PstI and BfaI primers. The structures of the PstI- and BfaI-adaptors are based on those described by Vos P. *et al.*, *Nucleic Acids Res.* 23: 4407-4414 (1995):

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5'-CTCGTAGACTGCGTACATGCA (SEQ ID NO: 7)
   3'-CATCTGACGCATGT (SEQ ID NO: 8)

5'-GACGATGAGTCCTGAG (SEQ ID NO: 3)
    3'-TACTCAGGACTCAT (SEQ ID NO: 4)
```

The corresponding PstI and BfaI non-selective primers have the following sequences:

5'-GACTGCGTACATGCAG (SEQ ID NO: 9)

5'-GATGAGTCCTGAGTAG (SEQ ID NO: 10)

The amplification step enriches the PstI-BfaI fragments over the large excess of BfaI-BfaI fragments. After amplification the fragments are fractionated on an agarose gel to eliminate the fragments smaller than 100 base pair, and cloned in an appropriate vector (e.g. TA cloning system; Invitrogen, Carlsbad, CA, U.S.A.).

Preparation of spotted micro-arrays with the cloned sample DNA fragments. The insert DNAs, from the two libraries of cloned PstI-BfaI sample fragments (obtained from the B73 and Mo17 inbred lines), are amplified from the clones using the non-selective PstI and BfaI primers. Following purification and concentration, the amplicons are arrayed as described in Example 1. A total of 20,000 (i.e. 10,000 from each library) candidate probe DNAs are spotted.

Micro-array hybridization and selection of candidate ESP-fragments. From genomic DNA of the inbred lines B73 and Mo17 four different sets of PstI/BfaI-digested amplified DNA are prepared. An alternative pair of adaptors and non-selective amplification primers are used for this:

5'-GAGCATCTGACGCATGTTGCA (SEQ ID NO: 11)
3'-GTAGACTGCGTACA (SEQ ID NO: 12)

5'-CTGCTACTCAGGACTG (SEQ ID NO: 13)
3'-ATGAGTCCTGACAT (SEQ ID NO: 14)

5'-CTGACGCATGTTGCAG (SEQ ID NO: 15)

5'-CTACTCAGGACTGTAG (SEQ ID NO: 16)

The sample fragments are amplified either as such or after digestion with one of three alternative probing enzymes, MseI, Tsp509I and AluI. As probing enzymes many different tetracutter or pentacutter enzymes can be used. Because plant

DNA has a high AT content, the preferred enzymes are those that have an AT bias in their recognition sequence. Alternatively, mixtures of two or more tetracutter or pentacutter enzymes can be used.

For each of the B73 samples, a Cy3(green)-labeled PstI primer is used, whereas the Mo17-derived fragments are amplified with a Cy5(red)-labeled PstI primer (refer to Example 1). Different hybridization solutions are then prepared by mixing equal amounts of the uncleaved, MseI-cleaved, Tsp509I-cleaved, and AluI-cleaved samples of both inbred lines. Each of the 4 mixes is allowed to hybridize to the micro-arrays. Analysis of the scanned images involved normalization using the multitude of probes on the arrays that detect monomorphic fragments. Figure 8 shows a false-color representation of the idealized results of the present experiment using a fictitious array of probes.

Analysis reveals that candidate ESP fragments are readily identified by scoring the probes that hybridize with only one of the two inbred line sample DNAs after cleavage with the probe enzyme (Figure 8). The quantitative analysis allows us the use of an unambiguous cut-off threshold of 10-fold difference in the normalized signal intensities for scoring ESPs. It should be pointed out that the assay identifies both bona fide ESPs and polymorphisms in the sampling enzyme sites. Most of the latter polymorphisms result in a marked hybridization difference with the sample DNAs not cleaved with the probe enzyme (see Figure 8). Analysis of 180 probes reveals that roughly 6% of the sample fragments carry ESPs for MseI, Tsp509I, or AluI, in accordance with the expected ESP mutation frequency. The analysis of 20,000 cloned probe fragments is thus expected to yield a total of 1,200 fragments carrying ESPs for the three probe enzymes tested. By using additional tetracutter and pentacutter enzymes (see Table I), the fraction of ESP carrying fragments may be as high as 25%, amounting to 5,000 ESPs.

Of all probes that exhibit a differential hybridization with the cleaved sample DNAs, only those in which the recognition site for the probing enzyme is present were retained for development of a corn micro-array. Sequence determination of these probe-fragments reveals the position of the recognition site for the probe enzyme. Thus, we retained only those probes that failed to give a signal with the

cleaved sample DNA from the same inbred line from which they were isolated. Such probes exhibit the hybridization pattern shown in the Table here below and are marked with an arrow in Figure 8.

	B73/Mo17 (Cy3/Cy5) normalized hybridization signal	
	Undigested	MseI/Tsp509I/AluI-digested
B73-probes	~1	< 0.1
Mo17-probes	~1	> 10

Step 2. Development of a corn ESP micro-array

Sequencing of the candidate ESPs and design of marker specific primers. Clones corresponding to the probes that yield the desired hybridization pattern (Figure 8) are sequenced. The majority of the insert DNAs derived from these clones contain a single recognition site for the probing enzyme. For each unique candidate ESP, two specific PCR primers, flanking the restriction site, are designed.

In addition, the sequence of a limited set of probes that yielded invariant hybridization signals is also determined. PCR primers targeting these monomorphic sequences are included as references; they are used to calibrate the hybridization signals.

Validation of the candidate ESPs and fabrication of corn micro-arrays. The candidate ESPs, identified under step 1, are subjected to a confirmatory experiment using the format-III approach. First, four pre-amplification reactions are performed with a single primer pair and using the PstI-BfaI fragments, undigested or digested with either one of the three probing enzymes, as template material. These amplification reactions reduce the complexity of the DNA under study by more than two orders of magnitude while at the same time generating a large enough amount of material for the subsequent multiplex marker-specific PCRs. The pre-amplifications are then used for the PCR rescue of each of the characterized candidate ESPs using dedicated primer couples [refer to Wang, D. G. *et al.*, *Science* **280**:1077-1082 (1998)]. Particular sets of the ESP-specific primers that amplify the same type of ESP (i.e. ESPs for one particular probing enzyme) are combined in a single reaction, together

with the appropriate pre-amplification material as template. One of the ESP-specific primers is either Cy3- or Cy5-labeled; the other remained unlabeled. The Cy3-primers are used for the multiplex amplification of the DNA that had previously been digested with a probing enzyme, whereas the Cy5-primers are used with undigested control DNA. The PCR products from the various multiplex reactions performed on both digested and undigested DNA were pooled together to obtain a single hybridization mixture per starting DNA. The B73 and Mo17 derived material was analyzed in parallel experiments. The set of ESP-specific unlabeled PCR primers served as hybridization probes and was arrayed in the same way as amplification products. Conditions used are similar to those previously described for hybridization against oligonucleotide probes and are readily determined by one of ordinary skill in the art.

Direct comparison of the normalized Cy3 and Cy5 hybridization signals allows determination of the allelic state of the endonuclease target site in B73 versus Mo17. Primer pairs that do not allow unambiguous allele calling or that do not confirm the candidate ESPs identified with PstI-BfaI sampling (refer to step 1), are not retained for further work.

Step 3. Genetic analysis of a B73/Mo17 recombinant inbred population and of segregating populations

Genetic analysis of a B73/Mo17 inbred population. A collection of recombinant inbred lines derived from a cross between B73 and Mo17 is publicly available and provides a most useful set of lines for verifying and mapping the collection of ESP markers. The advantage of recombinant inbred lines over segregating populations is that each inbred line contains a different set of homozygous chromosome segments derived from either parent line. Consequently each ESP will be scored as either present or absent. Preparation of the sample DNAs and hybridization against the arrayed probes are performed as described under step 2. The experiment will, in the first place, allow the testing of selected ESPs in over 100 measurements; the results will result in the development of a second generation system that will only detect the most consistent ESPs. In addition, the linkage analysis of the segregation data will allow the construction of a fine genetic map of the markers. Finally, based on the

mapping data, an ordered ESP micro-array is developed for corn.

Genetic analysis of segregating populations. While isolated from two inbred lines, it is anticipated that the above-mentioned ordered ESP micro-arrays will detect sufficient genetic polymorphism in other corn lines to be useful for marker assisted selection. To demonstrate the applicability, one could either chose a segregating F2 population or a back-cross population. Sample preparations and hybridizations are again performed as described under step 2. In this experiment, the ESP markers must be scored quantitatively so as to differentiate between heterozygosity and homozygosity. Because only the most consistent markers are retained, a two-fold difference in signal intensity is easily monitored. The approach used consists of normalizing the hybridization signal intensities and then applying a mixture model analysis on the normalized data. This statistical approach consists of determining whether the relative signal intensities can be grouped into three discrete classes, corresponding to respectively homozygous present, heterozygous and homozygous absent. ESP markers that do not fulfill this criterion should be eliminated from the analysis.

Example 3

Human Genetic Analysis Using the Format-I RAA

This example illustrates the application of the method of the invention for genome-wide genetic analysis in humans. Human is an example of a high complexity genome (size ~3,000 Mb) combined with a very low level of genetic variability. Single nucleotide differences between pairs of allelic sequences from different individuals occur approximately once in every 1000 basepairs; in the population at large, the frequency may be in the order of 1:300. As with Arabidopsis, such a low frequency necessitates the use of a selection procedure for the isolation/enrichment of the rare ESP-harboring fragments. In this example a batch-wise hybridization is used to accomplish this.

Based on the known mutation frequencies, it can be estimated that the ESP frequency for a tetracutter-probing enzyme is in the order of 1 in 125 recognition sites. This low level of genetic variation, in combination with the sensitivity of micro-

array hybridization, limits the number of ESPs that can be detected in a single assay (typically ranging from a few hundred to one thousand, a few thousand at the most). These limitations can, to a certain extent, be overcome by choosing probing enzymes that recognize tetranucleotide sites containing a CpG dinucleotide. Indeed, it is well documented that a substantial fraction (≥ 25%) of the nucleotide substitutions in the human genome result from C → T transitions in CpG dinucleotides. Such CpG dinucleotides represent mutational hotspots in vertebrates because a large fraction of the cytosines are methylated and subsequently mutate to thymine by deamination. It is estimated that the mutation frequency of methylated cytosines is 6 to 8-fold higher than average. Hence probing enzymes that cleave CpG-containing recognition sites will yield ESPs at correspondingly higher frequencies, estimated at ~5%. However, the adverse consequence of the high mutation rate is that CpG is relatively rare in mammalian DNA, occurring with a frequency of 1 in 100 nucleotides [Wang, D. G. *et al.*, *Science* **280**:1077-1082 (1998)] instead of 1 in 16. Likewise the frequency of CpG-containing tetranucleotide sites is 1 in ~1600 instead of 1 in 256 bases. To compensate for this, a probe endonuclease reagent can be used, comprising of two or more of the following complementary restriction enzymes: TaqI (TCGA), MspI (CCGG), MaeII (ACGT), and HinPI or HhaI (GCGC). It should be noted however that cleavage by MaeII as well as the isoschizomers HinPI and HhaI is blocked by methylation of the cytosine residue (C^5) within the CpG dinucleotide. These enzymes will thus only cleave at a fraction of their sites, namely the non-methylated sites. Analysis of the large amount of publicly accessible human genomic DNA sequence shows that the cocktail of the 4 enzymes will cleave once in every 400 bp on average. The total number of sites in the genome is thus in the order of 7.5 million. Assuming that the ESP frequency is 5%, the enzyme cocktail has the potential of detecting ~375,000 ESPs. In addition to using combinations of restriction endonucleases, one may also use reaction conditions that decrease the cleavage specificity. Such a strategy has been applied to obtain a restriction endonuclease reagent, designated CGaseI, that is capable of cleaving DNA at CpG dinucleotides [Mead D. *et al.*, WO 94/21663]. This CGaseI restriction endonuclease reagent may be particularly useful for the analysis of human polymorphisms using the methods of the present invention.

The example described below illustrates the approach in a limited scale assay, which characterizes the human ESPs within CpG-containing tetranucleotide recognition sites using the sampling enzyme combination PacI - BfaI. The rare cutter PacI is estimated to have only about 50,000 cleavage sites in the human genome; the frequent cutter BfaI will generate two fragments per PacI site. The enzyme combination will, therefore, create a moderately complex set of 100,000 PacI-BlaI target fragments. This fragment set captures a sizable number of CpG-containing restriction sites, estimated in the order of 40,000. Assuming a 5% ESP frequency, the number of detectable ESPs is in the order of 2000. It should be stressed that many different sampling enzyme combinations can be used and that thus a substantial fraction of the ~375,000 ESPs located within NCGN-type restriction sites can be monitored.

The procedure outlined in this example comprises the following steps:

(1) Development of a set of candidate PacI-BfaI ESP fragments

(2) Genetic analysis of humans using ESP probe fragments

Step 1. Development of a set of PacI-BfaI probe fragments

A mixture of sample fragments, derived from various individuals in the population, can be divided in three classes with respect to sites for the probing enzyme: monomorphic fragments that are devoid of a cleavage site, fragments that are always cleaved, and fragments that carry one polymorphic recognition site. Fragments that are digested will be referred to as S+ fragments and fragments lacking the site as S- fragments. Polymorphic ESP fragments will thus be the only fragments present in both the S+ and S- population of sampling fragments. This forms the basis for their selection by batch-wise hybridization: only ESP fragments are capable of annealing when mixing the S+ and S- fragment collections. The hybridization-selection can be performed in two different, reciprocal ways: either the S+ fragments can be used to retrieve the matching S- fragments, or S- fragments are used to collect the complementary S+ sampling fragments. In one approach, the selected candidate ESP fragments may be isolated by cloning, arrayed, and subsequently validated by testing various sample DNAs (e.g. the various sample DNAs used as starting material for the hybridization-selection). Candidate ESP probe fragments that appear to detect

monomorphic sample fragments may either be removed from the array or retained as control elements on the array. An alternative approach consists of performing the two reciprocal hybridization-selections, cloning the selected fragments, and identification of ESPs by means of matching S+ and S- fragments. The latter strategy is outlined below.

(i) Preparation of S+ and S- fragments The preferred starting material is an equimolar mixture of genomic DNA from a number of representative individuals. Such individuals (ranging from 5 to 50) may be chosen from various CEPH (Centre d'Etude du Polymorphisme Humain) pedigrees [Wang, D. G. *et al.*, *Science* **280**:1077-1082 (1998)]. Following cleavage of the DNA mixture with the PacI/BfaI-combination of sampling enzymes, appropriate oligonucleotide adapters as described above are ligated to the fragment ends. This template DNA is divided in two aliquots and treated separately to prepare respectively the S+ and S- fragment mix. To prepare the S- fragment mix, the target DNA fragments are cleaved with the probing enzyme and then amplified. This will result in a mixture of fragments that do not contain sites for the probing enzyme. Furthermore, the S- fragment mixture may be prepared by using one biotinylated primer, such that the resulting PCR product can be captured onto a solid substrate, such as magnetic beads conjugated with streptavidin. S+ fragments are prepared by (1) amplifying the mixture of PacI-BfaI fragments, (2) digesting the PCR product with one of the four NCGN-recognizing enzymes, (3) ligating appropriate adapters to the ends generated by the probing enzyme (see EP 0 534 858, incorporated herein by reference), and (4) re-amplification of the resulting material using one primer that recognizes the probe enzyme adapter and one primer that recognizes one specific sampling enzyme adapter. Similar to the S- fragments, the amplification reaction can be performed making use of a biotinylated primer that matches the probe enzyme adaptor such that the S+ fragment mixture can be immobilized.

Two alternative pairs of PacI- and BfaI-adaptors, as well as corresponding non-selective primers are used; e.g. set I is used for the amplification of the S- fragments and set II for the preparation of S+ fragments:

Set I

5'-CTCGTAGACTGCGTACCCAT (SEQ ID NO: 17)
3'-CATCTGACGCATGGG (SEQ ID NO: 18)

5'-GACGATGAGTCCTGAG (SEQ ID NO: 3)
3'-TACTCAGGACTCAT (SEQ ID NO: 4)

5'-GACTGCGTACCCATTA (SEQ ID NO: 19)

5'-GATGAGTCCTGAGTAG (SEQ ID NO: 10)

Set II

5'-GAGCATCTGACGCATGGGAT (SEQ ID NO: 20)
3'-GTAGACTGCGTACCC (SEQ ID NO: 21)

5'-CTGCTACTCAGGACTG (SEQ ID NO: 13)
3'-ATGAGTCCTGACAT (SEQ ID NO: 14)

5'-CTGACGCATGGGATTA (SEQ ID NO: 22)

5'-CTACTCAGGACTGTAG (SEQ ID NO: 16)

The adaptor ligated to the ends generated by the NCGN-cleaving probing enzyme and the corresponding amplification primer have the following structures:

5'-GTCCTCATCGAGCATG (SEQ ID NO: 23)
3'-AGTAGCTCGTACGC (SEQ ID NO: 24)

5'-CCTCATCGAGCATGCG (SEQ ID NO: 25)

(ii) Hybridization-selection step(s) The S- fragment mix is hybridized to the biotinylated S+ fragments. Following hybridization, the biotinylated products are captured onto streptavidin-coated magnetic beads. The beads are repeatedly washed to remove all unhybridized fragments and thereafter the hybridized S- fragments are eluted. These are then reamplified with the PacI and BfaI primers and the hybridization-selection procedure is repeated at least once. Finally the amplified

fragments are cloned in an appropriate vector and a series of around 2,000 inserts are sequenced. To select a set of S+ fragments, this procedure is repeated in reverse using this time biotinylated S- fragment. Upon comparison of the S+ and S- sequences ESP fragments are readily identified as fragments having partially overlapping sequences and in which the S- fragment sequence shows a mutated NCGN restriction site at the internal boundary of the overlap. In this way, ≥500 ESPs are readily characterized.

Step 2. Genetic analysis of humans using ESP probe fragments

The sequence-verified ESP fragments are spotted on micro-arrays for genetic analysis of human sample DNA. For the preparation of this sample DNA, a pair of adaptors/primers is used that differs from those attached to the arrayed S- or S+ set of ESP fragments. From each individual, an undigested control sample and a probe enzyme digested test sample are prepared. These samples are labeled with Cy3 and Cy5, mixed and hybridized to the micro-arrays as described before. Alternatively, the hybridization mixture may be composed of differentially labeled test DNA and previously genotyped control DNA, both digested with the probing endonuclease. In both cases, the Cy3 (test/digested sample) and Cy5 (control/undigested DNA) signal intensities are normalized using a number of monomorphic control probes. The ratio of these normalized Cy3/Cy5 signals for each of the ESP probes, allows accurate determination of the allelic state of the sample at each polymorphic site (homozygous S+/S+, homozygous S-/S-, heterozygous S+/S).

The micro-array hybridization experiment may in the first place be performed with the sample DNAs, deriving from a collection of individuals, from which the ESP probe fragments were isolated. Such an experiment will, in the first place, confirm the polymorphic nature of the selected probe fragments and allow their testing in a multitude of measurements. The data will also yield information on the allele frequencies among an appreciable number of chromosomes.

Example 4

Human genetic analysis using format-II RAA

As described for corn in Example 2, the format-I ESP assay for human genetic analysis may be converted to a format-II or a format-III assay. Based on the sequence of the selected and experimentally validated ESP fragments, it is indeed possible to design a pair of dedicated, i.e. ESP-specific, PCR primers. Such primers can be combined in a number of parallel multiplex reactions, which are in turn combined to obtain the sample DNA [Wang, D. G. *et al., Science* **280**: 1077-1082 (1998)]. This sample DNA is hybridized against a micro-array of spotted S+ ESP fragments (*see* to Example 3). The experiment is set up such that the fluorescently labeled ESP-specific primer and the S+ sequences are located on opposite sides of the polymorphic site. Alternatively, the unlabeled ESP-specific amplification primers may be arrayed as hybridization probes. The development of a format-II or format-III assay need not be preceded by the identification of ESP fragments (using one of the methods described in the previous examples). In the present example, we describe the development of an RAA assay based on the sequence of previously discovered SNPs.

Close inspection of the known SNPs reveals that a significant percentage of them are associated with both the loss and gain of a restriction recognition site, i.e. each of two allelic sequences is associated with a different restriction recognition site. The single nucleotide substitution may inter-convert recognition sequences that are identical except for one nucleotide [e.g. PleI (GACTC) and HgaI (GACGC), HgaI and SfaNI (GATGC), SfaNI and BbvI (GCTGC)]. Alternatively, the allelic recognition sites may be partially overlapping [e.g. MaeII (ACGTg) and NlaIII (aCATG); in the latter case the inter-conversion depends on the nature of the upstream or downstream sequences). Such mutually exclusive restriction site allelism offers a distinct advantage. The RAA technique will normally only detect the allele that is devoid of a recognition site for the probing enzyme; therefore, determination of the zygosity requires careful calibration of the signal against that observed with undigested control DNA. When each allele is associated with the presence/absence of a restriction site, two parallel RAA-assays can be performed, each involving digestion with one of the alternative enzymes.

With such an assay, both alleles can be positively identified and the zygosity is readily determined. The two parallel assays are best performed in a two-color mode; one of the primers is differentially labeled (e.g. with Cy3 and Cy5 as described previously) such that the amplification reactions can be mixed and hybridized against a single array of probes.

We have systematically explored the SNP database of the Whitehead Institute for mutational changes that promote restriction site inter-conversions and have calculated their occurrence frequency. Two SNP-associated recognition site inter-conversions were found to occur at high frequency: MaeII -> NlaIII and HgaI -> SfaNI. In both cases the mutational changes converting one site into another are C→T (or G→A) transitions occurring in CpG dinucleotides. This finding is entirely consistent with the fact that this type of mutation occurs with a 6-8 times higher frequency than other nucleotide substitutions. Based on the number of SNPs found in the Whitehead database, we estimate the total number of SNPs in the human genome for the enzyme pairs MaeII/NlaIII and HgaI/SfaNI at respectively 30,000 and 15,000. These numbers are presumably somewhat overestimated since both MaeII and HgaI are susceptible to CpG methylation. Consequently the inter-conversion can only be measured at the non-methylated sites. Therefore, in practice, RAA assays designed on the basis of sequence data should be validated on a number of test samples. Assays in which no cleavage takes place at the CpG-containing site in none of the individuals tested, should be eliminated from the RAA bi-allelic marker systems.

The foregoing examples are illustrative of the invention and are not intended to be limit the scope of the invention as set out in the claims. All of the references cited herein are incorporated by reference.

WE CLAIM:

1. A method for detecting an endonuclease site polymorphism (ESP) in DNA, the method comprising:

(a) isolating sample DNA;

(b) deriving a set of concomitantly amplifiable target DNA fragments from the sample DNA;

(c) treating the target DNA fragments obtained in step (b) with a probe restriction endonuclease reagent;

(d) amplifying the probe restriction endonuclease reagent treated target DNA fragments of step(c);

(e) analyzing the DNA of step (d) to determine which target fragments are amplified and/or which target fragments are not amplified; and wherein target DNA fragments which are amplified lack a recognition site for the probe restriction endonuclease reagent and target fragments having a recognition site for the probe restriction endonuclease reagent are not amplified.

2. The method of claim 1 the concomitantly amplifiable target DNA fragment of step (b) are derived by treatment of the sample DNA with a sampling restriction endonuclease reagent.

3. The method of claim 2 wherein the concomitantly amplifiable DNA fragments of step (b) are derived from sample DNA by treatment of the sample DNA with a first and a second restriction endonuclease reagent.

4. The method of claim 3 wherein said first restriction endonuclease reagent has a recognition sequence of six or more nucleotides and the second restriction endonuclease reagents has a recognition sequence of four or fewer nucleotides.

5. The method of claim 3 or 4 wherein said concomitantly amplifiable target DNA fragments are derived by step wise treatment of said sample DNA with the

first and the second restriction endonuclease reagents.

6. The method of claim 1 further comprising preparing of PCR primers which flank the endonuclease site polymorphism (ESP) for use in amplifying said concomitantly amplifiable target DNA fragments.

7. The method of claims 1, 2, 3, and 4 wherein the concomitantly amplifiable DNA fragments are modified by ligation of adapters to both termini of said fragments, and wherein said adaptors are capable of serving as primers for amplification.

8. The method of claim 5 wherein the concomitantly amplifiable DNA fragments are modified by ligation of adapters to both termini of said fragments, and wherein said adaptors are capable of serving as primers for amplification.

9. The method of claim 1 wherein the probe restriction endonuclease reagent of step (c) has a recognition sequence comprising six or more nucleotides.

10. The method of claim 1 wherein the probe restriction endonuclease reagent of step (c) has a recognition sequence comprising four or more nucleotides.

11. The method according to claim 1 wherein the probe restriction endonuclease of step (c) has a recognition sequence of two nucleotides.

12. The method according to claim 1 wherein the order of the steps (b) and (c) are reversed or carried out simultaneously.

13. The method according to claim 1 wherein said endonuclease site polymorphism is an alteration in a concomitantly amplifiable target fragment giving rise to a nucleotide sequence that is recognized and cut by the probe restriction endonuclease reagent .

14. The method of claim 1 wherein said site polymorphism is an alteration in the nucleotide sequence of a concomitantly amplifiable target fragment which eliminates a recognition sequence for said probe restriction endonuclease reagent.

15. The method of claims 1, 2, 3 and 4 wherein said concomitantly amplifiable DNA fragments are amplified by a polymerase chain reaction.

16. The method of claim 5 wherein said concomitantly amplifiable DNA fragments are amplified by a polymerase chain reaction.

17. The method of claim 1 wherein amplified target fragments are identified by their ability to hybridize to cognate probe DNA fragments.

18. A method for obtaining probe DNA fragments for use in detecting endonuclease site polymorphisms, the method comprising:

(a) isolating sample DNA;

(b) deriving a set of concomitantly amplifiable target DNA fragments from the sample DNA;

(c) selecting from the target DNA fragments, probe DNA fragments having an endonuclease site polymorphism (ESPs) for the probe restriction endonuclease.

19. The method of claim 17 wherein said probe DNA fragments are derived by digestion of sample DNA with one or more sampling restriction endonuclease reagents.

20. The method of claim 18 wherein probe DNA fragments are derived by digestion of a pool of sample DNAs obtained from one or more individuals of a species.

21. The method of claim 18 wherein the probe DNA fragments are derived

by digestion of a pool of sample DNAs obtained from 10 or more individuals of a species.

22. The method of claim 18 wherein the probe DNA fragments derived by digestion of a pool of sample DNAs obtained from a pool of 50 or more individuals of species.

23. The method of any one of claims 19-21 wherein said species is selected from the group consisting of procaryotic species and eucaryotic species.

24. A method for obtaining probe DNA fragments for use in detecting endonuclease site polymorphisms (ESP) comprising preparing synthetic oligonucleotides based on the nucleotide sequence of amplifiable target DNA fragments containing endonuclease site polymorphism(s).

25. A method for producing a microarray of probe DNA the method comprising:

(a) isolating sample DNA;

(b) deriving a set of concomitantly amplifiable target DNA fragments from the sample DNA;

(c) selecting probe DNA fragments having restriction endonuclease site polymorphisms (ESPs) from the sample restriction endonuclease treated target DNA fragments of step (b); and

(d) arraying the probe DNA fragments obtained in step (c) on a solid substrate in a predefined region by attaching the fragments to the substrate.

26. The method of claim 24 wherein the DNA fragments of step (b) are obtained by treating sample DNA with one or more sample restriction endonuclease reagents.

27. The method of claim 24 wherein the said probe DNA fragments of step

(d) are synthetic oligonucleotides which correspond to the concomitantly amplifiable target DNA fragments derivable from said sample DNA and containing an endonuclease site polymorphism (ESP).

28. The method of claim 25, 26 or 27 wherein the solid support is selected from a group consisting of a planar solid support, a bead, a sphere and a polyhedron.

29. The method of claim 25 wherein the microarray comprises at least 2,000 probe fragments.

30. The method of claim 26 wherein the microarray comprises at least 2,000 sythetic ologonucleotides.

31. The method of claim 27 wherein the microarray comprises at least 2,000 probe fragments.

32. The method of claim 28 wherein the microarray comprises at least 2,000 probe fragments.

33. The method of claim 25 wherein the microarray comprises at least 20,000 probe fragments.

34. The method of claim 26 wherein the microarray comprises at least 20,000 sythetic ologonucleotides.

35. The method of claim 27 wherein the microarray comprises at least 20,000 probe fragments.

36. The method of claim 28 wherein the microarray comprises at least 20,000 probe fragments.

Figure 1

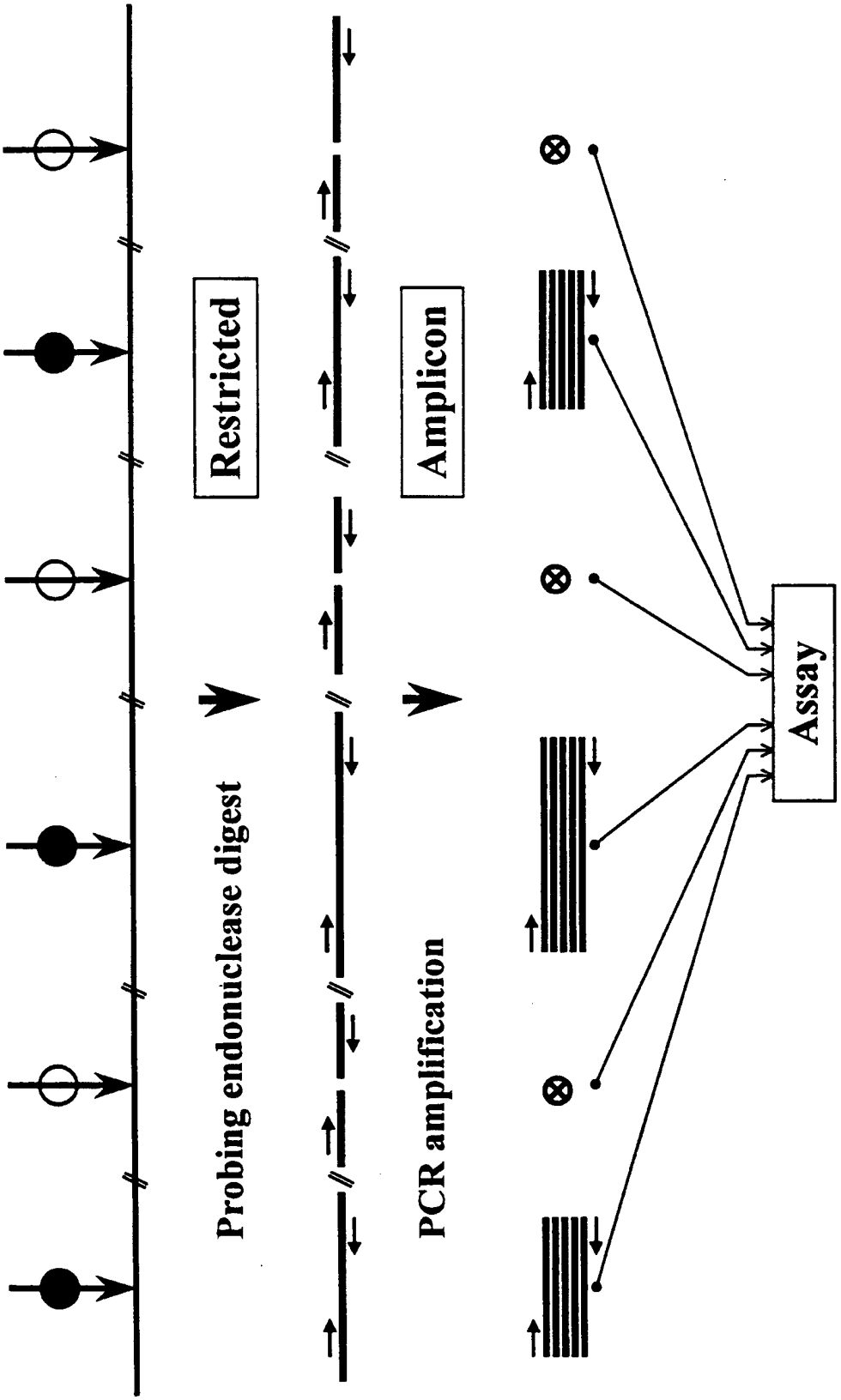

Restricted
Probing endonuclease digest
Amplicon
PCR amplification
Assay

Figure 2

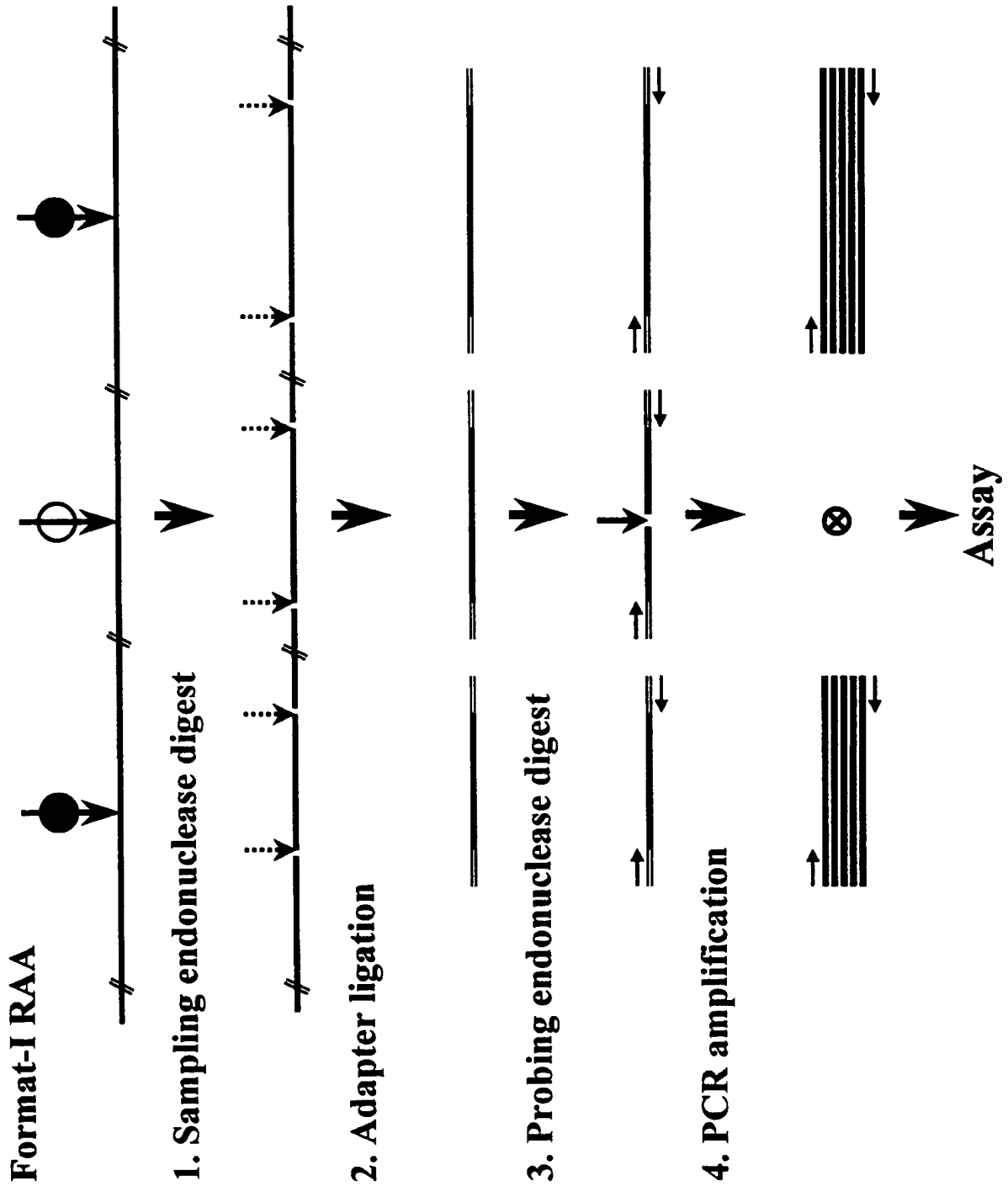

Format-I RAA
1. Sampling endonuclease digest
2. Adapter ligation
3. Probing endonuclease digest
4. PCR amplification
Assay

Figure 3

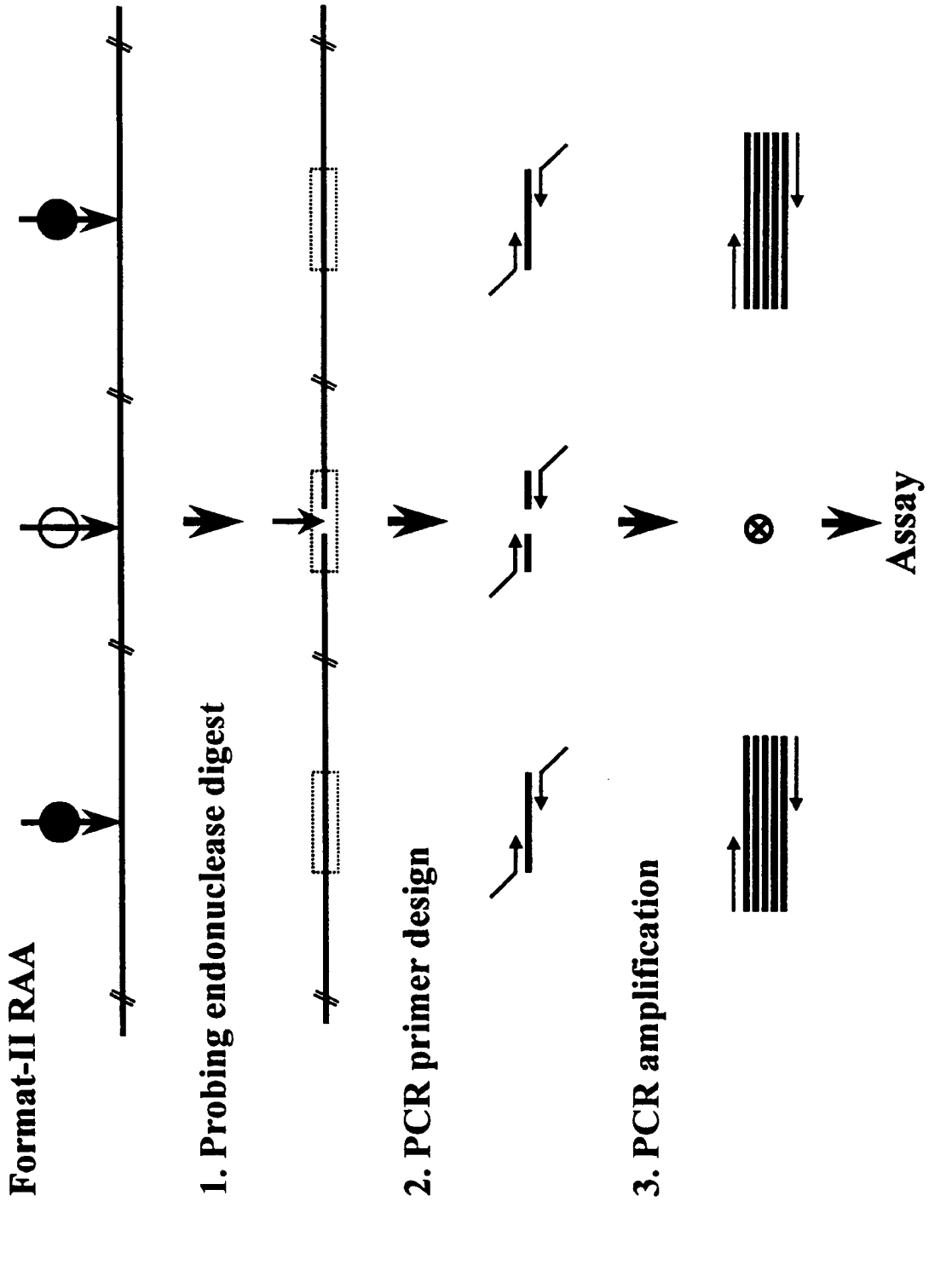
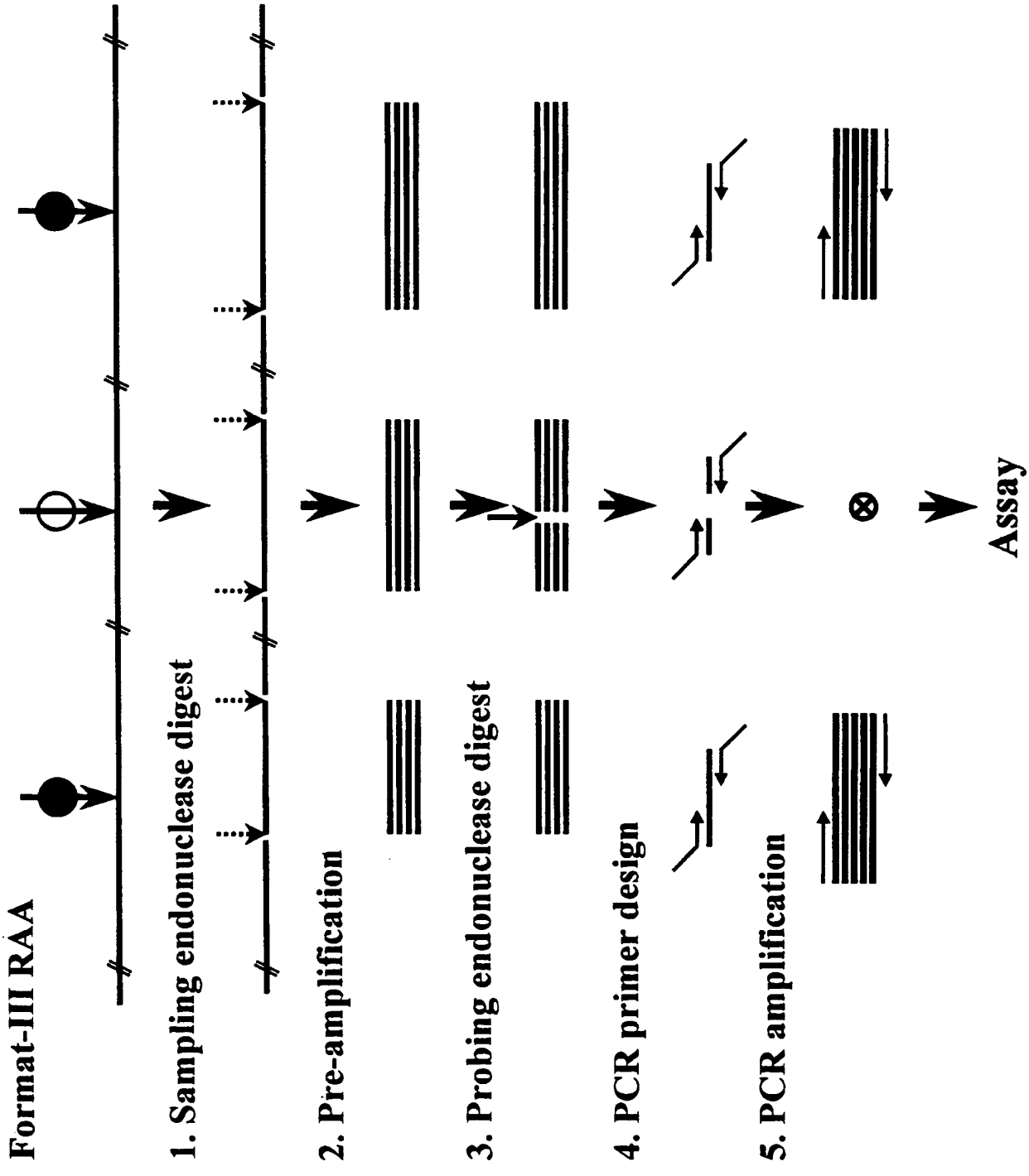

Format-II RAA
1. Probing endonuclease digest
2. PCR primer design
3. PCR amplification
Assay

Figure 4


Format-III RAA
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2. Pre-amplification
3. Probing endonuclease digest
4. PCR primer design
5. PCR amplification
Assay

Figure 5

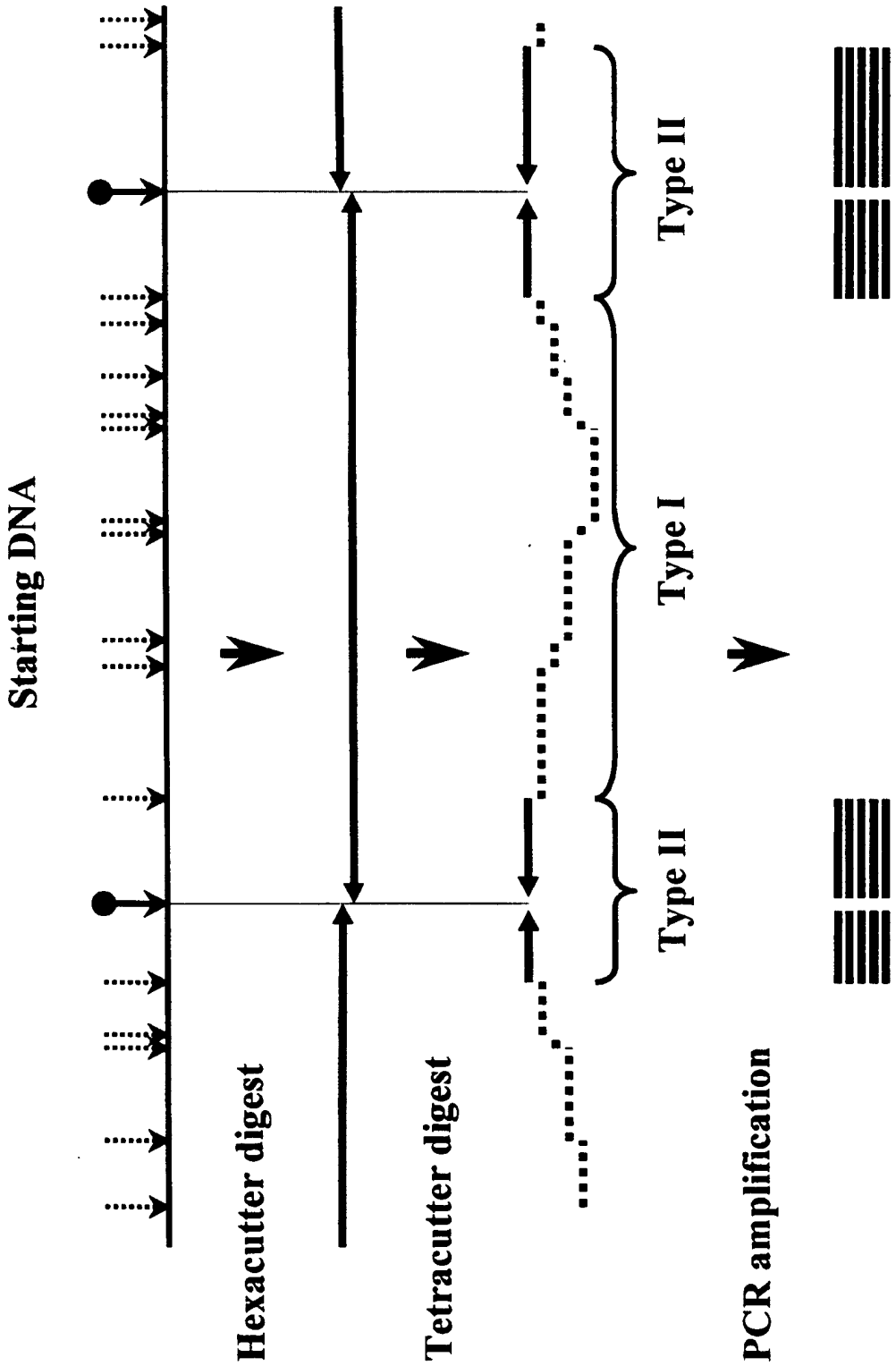
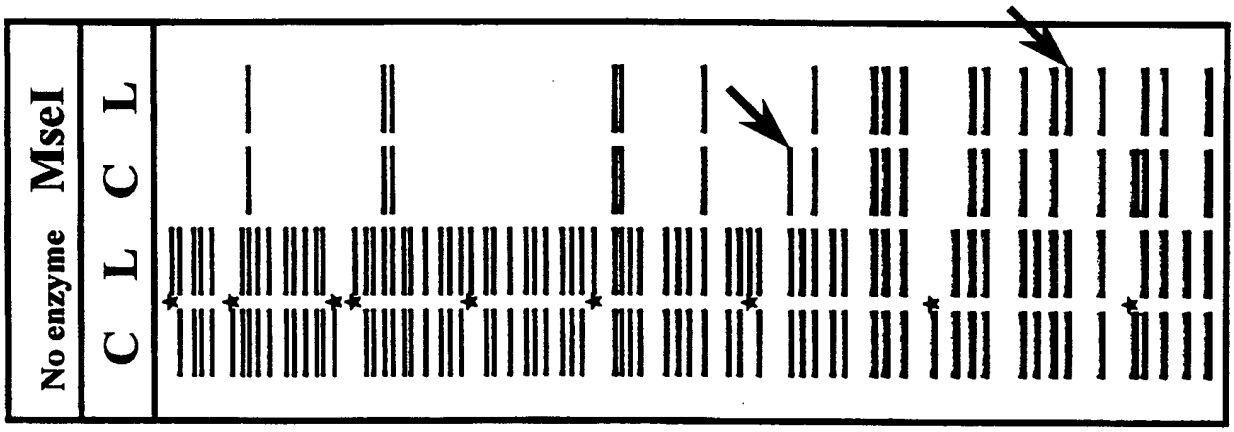

Starting DNA
Hexacutter digest
Tetracutter digest
PCR amplification
Type II
Type I
Type II

Figure 6



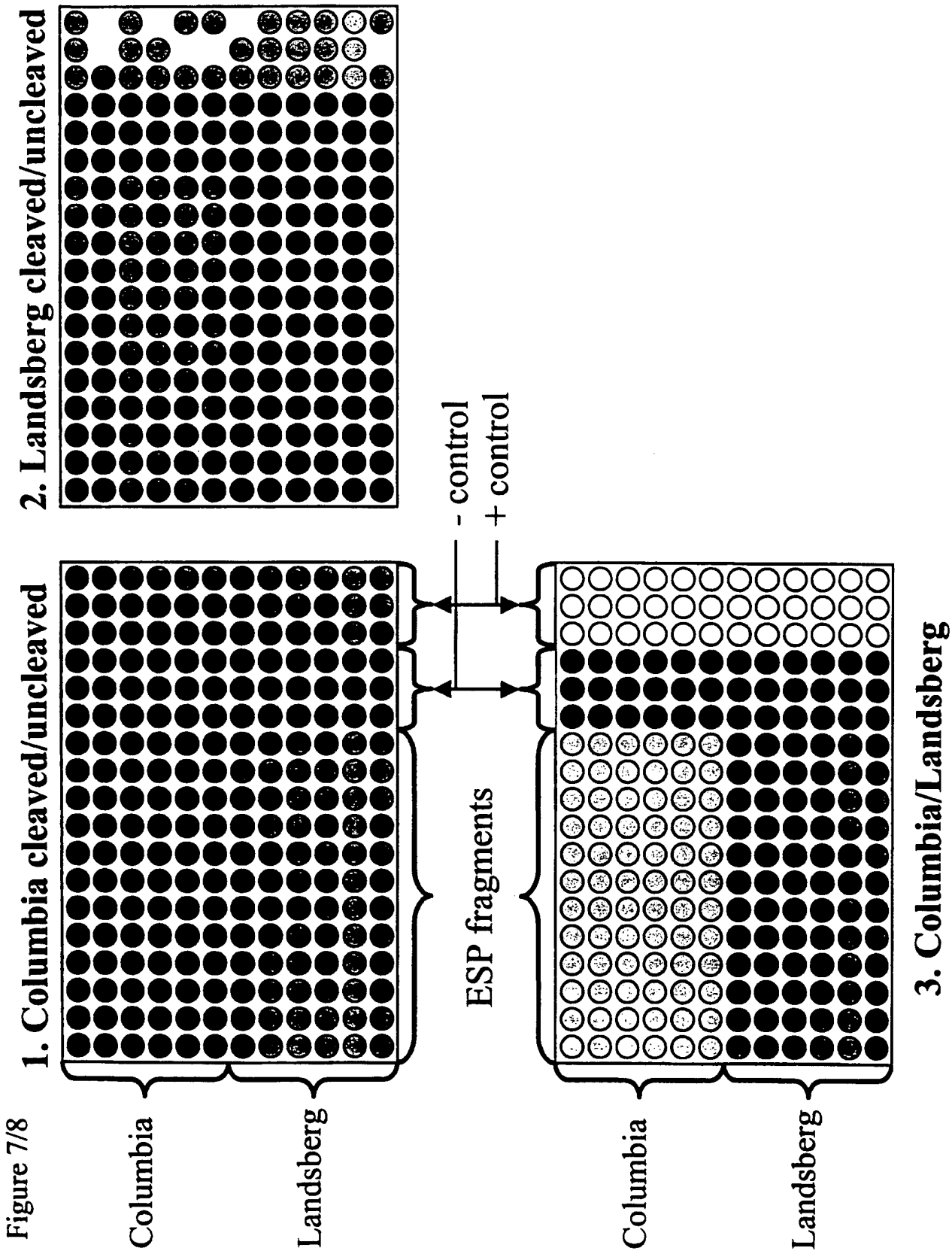
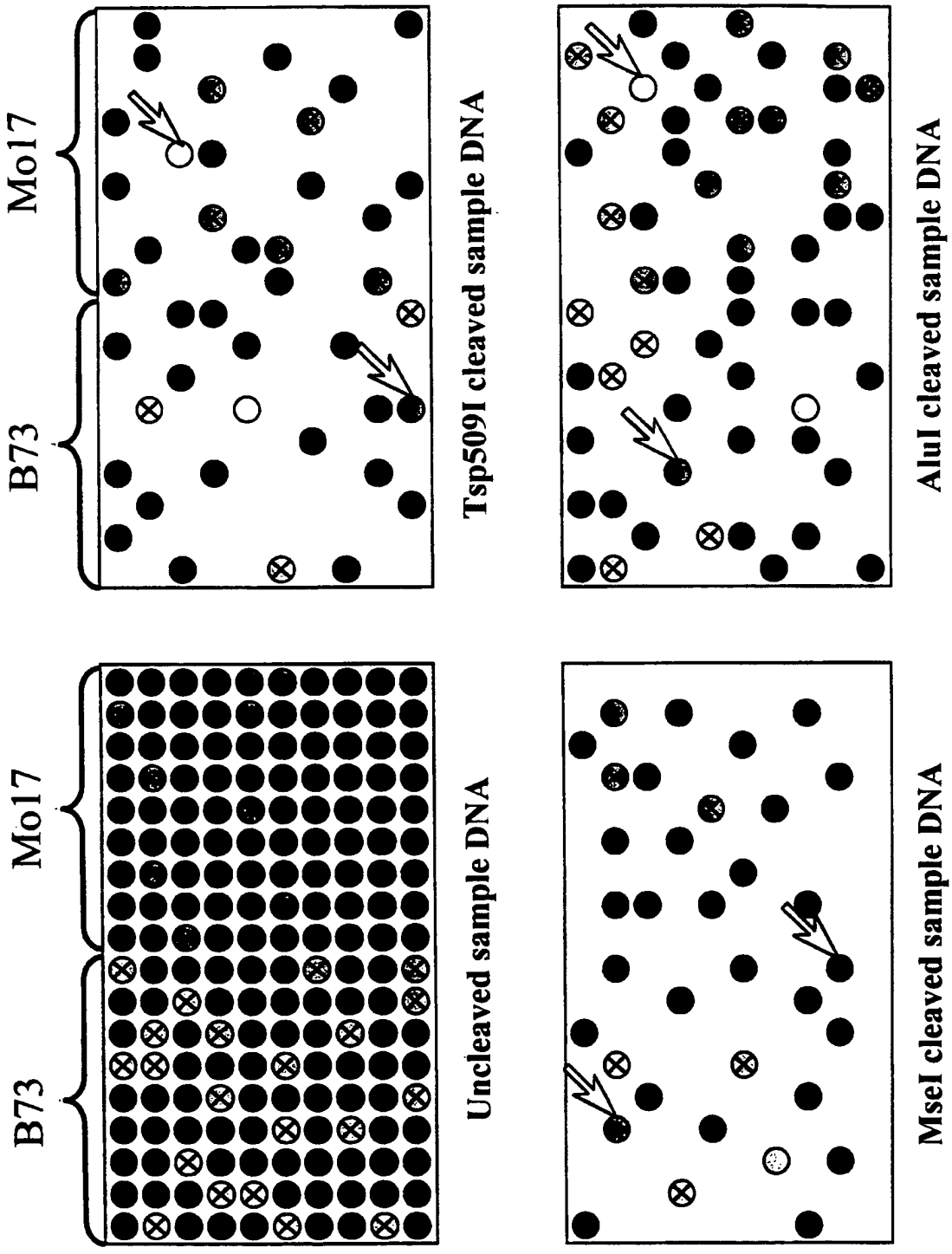

1. Columbia cleaved/uncleaved
2. Landsberg cleaved/uncleaved
- control
+ control
ESP fragments
Columbia
Landsberg
Columbia
Landsberg
3. Columbia/Landsberg

Figure 7/8

Figure 8/8


B73
Mo17
Uncleaved sample DNA
Tsp509I cleaved sample DNA
AluI cleaved sample DNA
MseI cleaved sample DNA

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<120> RESTRICTED AMPLICON ANALYSIS

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<140>
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<150> 60/107,293
<151> 1998-11-09

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/IB 99/01958

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 34015 A (PENN STATE RES FOUND) 18 September 1997 (1997-09-18) the whole document ---	1-24
X	WO 98 12352 A (GEN HOSPITAL CORP ;UNIV LELAND STANFORD JUNIOR (US)) 26 March 1998 (1998-03-26) the whole document --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	14/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Molina Galan, E

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US1998 GILAD SHLOMIT ET AL: "Identification of ATM mutations using extended RT-PCR and restriction endonuclease fingerprinting, and elucidation of the repertoire of A-T mutations in Israel." Database accession no. PREV199800093427 XP002139123 abstract & HUMAN MUTATION 1998, vol. 11, no. 1, 1998, pages 69-75, ISSN: 1059-7794	1-24
X	WO 91 17262 A (UNIV BRITISH COLUMBIA) 14 November 1991 (1991-11-14) the whole document	1,18-24
X	EP 0 534 858 A (KEYGENE NV) 31 March 1993 (1993-03-31) cited in the application the whole document	18-24
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-/—

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/IB 99/01958

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WANG D G ET AL: "Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 280, 1 January 1998 (1998-01-01), pages 1077-1082, XP002089398 ISSN: 0036-8075 cited in the application the whole document	25-36
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna al Application No
PCT/IB 99/01958

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		DE 69507646 T	16-09-1999
		EP 0804618 A	05-11-1997
		JP 10509594 T	22-09-1998
		NZ 298236 A	28-01-1999
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		WO 9615139 A	23-05-1996

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68 — A2

(11) International Publication Number: **WO 00/28081**

(43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/IB99/01958

(22) International Filing Date: 9 November 1999 (09.11.99)

(30) Priority Data:
60/107,293 9 November 1998 (09.11.98) US

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ZABEAU, Marc [BE/BE]; Onafhankelijkheidslaan 38, B–9000 Gent (BE). STANSSENS, Patrick [BE/BE]; Constant Permekelaan 48, B–9830 St. Martens Laten (BE).

(74) Agent: DE CLERCQ, Ann; Ann De Clercq & Co B.V.B.A., Brandstraat 100, B–9830 Sint–Martens–Latem (BE).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RESTRICTED AMPLICON ANALYSIS

(57) Abstract

The present invention generally provides a method which facilitates the detection of polymorphisms (or mutations). The method is directed to the analysis of so–called endonuclease site polymorphisms (ESPs) that result in the gain or loss of a restriction endonuclease site. In essence, the ESP is probed with the restriction endonuclease reagent prior to amplification, whereby amplification is prevented and consequently no signal is observed when cleavage takes place. Unambiguous allele calling is performed by comparing the signals obtained with and without cleavage with the restriction endonuclease reagent. The method is particularly useful for multiplex genotyping, involving the parallel analysis of large numbers of single nucleotide polymorphisms. Preferred methods for detecting the amplicons involve hybridization to an arrayed or otherwise identifiable set of cognate probe fragments or oligonucleotides.

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RESTRICTED AMPLICON ANALYSIS

Field of the Invention

The present invention generally provides a method which facilitates the detection of polymorphisms (or mutations). The method is directed to the analysis of so-called endonuclease site polymorphisms (ESPs) that result in the gain or loss of a restriction endonuclease site. In essence, the ESP is probed with the restriction endonuclease reagent prior to amplification, whereby amplification is prevented and consequently no signal is observed when cleavage takes place. Unambiguous allele calling is performed by comparing the signals obtained with and without cleavage with the restriction endonuclease reagent. The method is particularly useful for multiplex genotyping, involving the parallel analysis of large numbers of single nucleotide polymorphisms. Preferred methods for detecting the amplicons involve hybridization to an arrayed or otherwise identifiable set of cognate probe fragments or oligonucleotides.

Background of the Invention

Molecular approaches for genetic analyses trace the nucleotide sequence variation that occurs naturally and randomly in the genomes of all living species. Knowledge of the DNA polymorphisms among individuals and between populations is important in understanding the complex links between genotypic and phenotypic variation. In the absence of complete data about sequence variation, one relies on the ability to identify 'nearby' markers that allow to infer the location of certain relevant loci or causal sequence variations. The informativeness of the marker depends on the magnitude of the linkage disequilibrium. Markers can be used in linkage studies to search for candidate genes and in association studies to identify the functional allelic variation on candidate genes that influence inter-individual variation.

The vast majority of sequence variation consists of nucleotide substitutions, often referred to as single nucleotide polymorphism's (SNPs), resulting from mutations that have accumulated during evolution. Most of these nucleotide

changes are genetically silent; i.e., they have no measurable biological effect, but provide an immense reservoir of variation in DNA structure. Most methods for genetic analysis used today rely on the detection of nucleotide sequence variation which can be measured by DNA fragment analysis using electrophoretic separation, in which DNA fragments are fractionated based on size or conformation. Occasionally the nucleotide sequence variation will affect either the presence of the DNA fragment or its mobility. In this way the primary nucleotide sequence variation will give rise to easily detectable DNA fragment polymorphism. Since polymorphic DNA fragments are derived from precise locations on the organism's genome, they can serve as reliable genetic markers, or landmarks to identify and locate genes.

A host of assays to detect DNA polymorphisms, and SNPs in particular, have been developed. In some of these assays (*e.g.*, RFLP [Botstein, D., White, R.L., Skolnich, M., Davis, R.W., *Am. J. Hum. Genet.* 32:314-331 (1998)], CAPS [Konieczny, A. Ausubel, J.F., *Plant J.* 4:403-410 (1993)], dCAPS [Neff, M.M. Neff, J.D., Chory, J., Pepper, A.E., *The Plant Journal* 14:387-392 (1998)], PIRA [Steinborn, R., Muller, M., Brem, G., *Biochim. Biophys. Acta* 1397:295-304 (1998)]), restriction enzymes are used to detect polymorphic nucleotide sequences that affect cleavage. The specificity of restriction enzymes is such that they exhibit a unique sensitivity to detect single nucleotide differences occurring in their recognition sites. The principal strengths of restriction enzyme-based genetic analyses are the ease of use and the robustness of the assays. In the majority of the cases, the restriction site polymorphism is used to detect known, previously identified SNPs and the assay consists of any electrophoretical fragment analysis. In one report, the allelic variation is detected in a solid-phase ELISA-type setting [Truett, G.E., Walker, J.A., Wilson, J.B., Redmann, S.M. Jr., Tulley, R.T., Eckardt, G.R., Plastow, G., *Mamm. Genome* 9:629-632 (1998)].

In WO 91/17269, Lerner *et al.* describe a different method for mapping a eukaryotic chromosome by restriction endonuclease mapping of discrete DNA sequences which are complementary to a region of a eukaryotic chromosome.

Vos *et al.*, *Nucl. Acids Res.* 23:4407-4414 (1995) and EP 0 534 858 describe a technique for DNA fingerprinting called AFLP which is based on the

selective polymerase chain reaction based application of restriction fragments of a digest of genomic DNA. The application reaction depends on the use of primers that extend into restriction fragments amplifying only those fragments in which prior extensions match the nucleotide sequence flanking the restriction sites.

Another method utilizing DNA amplification steps is set out in Williams *et al.*, *Nucl. Acids Res.* **18**:6531-6535 (1990), who describe a DNA fingerprinting method termed random amplified polymorphic DNA.

DNA amplification fingerprinting was described by Caetano Anolles in *Bio/Technology* 9:553-557 (1991). Still another fingerprinting technique called arbitrarily primed PCR was described in Welsh *et al.*, *Nucl. Acids Res.* **18**:7213-7218 (1990) and Welsh *et al.*, *Nucl. Acids Res.* **19**:861-866 (1991).

In WO 94/11530, Cantor *et al.* describe materials and methods for position and sequencing by hybridization. Cantor *et al.* also describe methods for creating assays of DNA probes useful in the practice of their method.

The major shortcoming of the current methods of genetic analysis is the limited resolution of the DNA fragment analysis systems, namely the number of DNA fragments that can be separated in a single assay. Generally the fractionation resolution ranges from tens to a couple of hundred DNA fragments, at the most. Consequently, current genetic analysis methods are limited to a few hundred to a thousand genetic markers. While this resolution has been sufficient for analyzing simple genetic traits determined by single genes, the analysis of complex traits, which is now being undertaken and which involve general or many different genes, will require the analysis of a much larger number of genetic markers. It is anticipated that such studies will require from a few thousand to possibly several hundred thousand genetic markers. Although this could conceivably be accomplished by performing many parallel assays, such scaling up will be cost- and labor prohibitive.

A technology that has great potential and which is generating widespread interest in the so-called micro-array technology (DNA chips). In general, these methods are based on measurement of the hybridization of DNA sequences in solution to probe sequences that are arrayed on a solid surface. When assaying nucleotide polymorphisms, the detector relies on the small differences in hybridization efficiency

between two different DNA sequences. In one format, fluorescently labeled sample DNA is hybridized to dense arrays of probe nucleic acids, sequence-specific hybridization signal is detected by scanning confocal microscopy, and DNA variants scored as (predictable) differences in the hybridization pattern. The micro-arrays are fabricated either by in-situ light-directed oligonucleotide synthesis [Fodor, S.P.A. *et al., Science* **251**: 767 (1991)] or by spotting DNA (off-chip synthesized oligonucleotides or PCR fragments) in an automated procedure. The technology has already been demonstrated in the scoring of mutations in mitochondrial DNA [Chee, M. *et al., Science* **274**: 610-614 (1996)], the HIV genome [Lipshutz, R.J. *et al., Biotechniques* **19**: 442-447 (1995)], the CFTR cystic fibrosis gene [Cronin, M.T. *et al., Human Mut.***7**: 244-255 (1996)], the BRCA1 breast cancer gene (Hacia, G.H. *et al., Nat. Genet.* **14**: 441-447 (1996)] as well as the entire yeast genome [Winzeler, E.A. *et al., Science* **281**:1194 (1998)]. In comparison with most other assays, micro-arrays provide a platform for high-throughput, massively parallel polymorphism detection.

A major disadvantage with the use of microarrays relates to the complexity of the hybridization reaction. The detection relies on the very small difference in hybridization of DNA sequences differing by only one nucleotide. In general, a set of 4 oligonucleotides, differing only in the identity of the central base, is synthesized for each position in the target sequence that has to be interrogated. In practice, the number of oligonucleotides needed to correctly genotype one SNP is much larger, involving up to 56 different oligonucleotides spanning the variable base [Wang *et al., Science* **280**: 1077-1082 (1998)]. The degree of redundancy is also dramatic if one wants to screen the target DNA for all possible mutations; the design then includes overlapping oligonucleotide-sets that are offset by one base (a process known as tiling). It should be noted that the detection of SNPs by hybridization to arrays depends on the use of short oligonucleotide probes. With longer probes such as DNA fragments in the size range of 50 to 500 base pairs or larger, it is not possible to distinguish the SNP alleles.

Summary of the Invention

The present invention is directed to methods for genotyping polymorphisms that result in the gain or loss of an endonuclease cleavage site. Such polymorphisms are referred to hereinafter as endonuclease site polymorphisms (ESPs). Polymorphisms detectable according to the methods of the present invention include single nucleotide polymorphisms (SNPs). The methods of the present invention exploit the high discriminatory power of restriction enzymes in a "Restricted Amplicon Assay" (RAA) which generally comprises the following steps (*see* Figure 1):

(a) isolating sample DNA;

(b) deriving a set of target DNA fragments, said set of target fragments comprising concomitantly amplifiable target DNA fragments from the sample DNA;

(c) treating the target DNA fragments obtained in step (b) a probe restriction endonuclease reagent;

(d) amplifying the amplifiable probe restriction endonuclease reagent treated target DNA fragments of step(c); and

(e) analyzing the DNA of step (d) to determine which target fragments are amplified and/or which target fragments are not amplified; and wherein amplified target fragments lack a recognition site for the probe restriction endonuclease reagent and target fragments having a recognition site for a probe restriction endonuclease reagent are not amplified.

In one aspect, the present invention is directed to RAA-methods, which comprise the preparation of concomitantly amplifiable DNA segments by digestion of the starting DNA with one or more restriction endonucleases, collectively referred to herein as sampling enzymes. This method is herein referred to as format-I RAA and is diagrammed in Figure 2. The digested starting DNA may be further modified at its termini by the addition of adapters, which may serve to prime an amplification reaction (see Figure 2). Once sample DNA is obtained, it is treated with a different restriction enzyme, the probing enzyme also referred to as a probe restriction endonuclease reagent. A combination of probing and sampling enzymes are chosen such that a

substantial fraction of the sample fragments contain a single recognition site for the probe endonuclease reagent. In general, probe enzymes used with format-I RAA preferably have as a recognition site a nucleotide sequence of less than six nucleotides.

In another aspect, the present invention is directed to methods for format-II RAA for the detection of ESPs, as diagrammed in Figure 3. Format-II RAA operates on the same principal as format-I RAA except that the sample amplicons need not be DNA fragments, but are rather defined regions of a genome amplifiable with specific primer pairs. The amplicons of the format-II RAA are identified on the basis of sequence data; e.g. the sequence of ESP-containing restriction fragments identified using format-I RAA method or otherwise known SNPs affecting endonuclease cleavage sites. In format-II RAA, the test DNA to be analyzed is treated with a probe restriction endonuclease reagent, followed by the concomitant amplification of regions of the treated DNA (amplicons) using predetermined primers using, for example, the polymerase chain reaction as described herein. The analysis of the amplification products then proceeds as described in the format-I RAA methods described herein. As with format-I RAA, an ESP is genotyped by the presence or absence of a recognition site for the probe restriction endonuclease reagent.

In yet another aspect, the present invention is directed to methods for format-III RAA. In essence, format-III RAA consists of a combination of the format-I and format-II approaches. One of such combinations is diagrammed in Figure 4. Test DNA, digested or not with a probe endonuclease reagent, is sampled with a pair of endonuclease reagents and the resulting fragments are co- as described in the format-I assay amplified (this step is referred to as the pre-amplification step). These pre-amplification mixtures are, in turn, used as templates for a format-II type of PCR reaction in which multiple ESP-containing regions are selectively co-amplified using specific primer sets. The analysis of the amplification products then proceeds as described before. The advantages of format-III RAA are that the stepwise amplification facilitates the multiplex PCR of the ESP-specific amplicons and lowers the amount of starting material required to interrogate all the ESPs.

Arrays, or microarrays of probe DNA wherein the probe DNAs are useful in the detection of ESPs are also encompassed by the present invention.

Informative probe DNAs are prepared and identified as described in detail below and are then attached to a substrate for use in the hybridization reactions with concomitantly amplifiable DNA after treatment with a probe restriction endonuclease reagent and subsequent amplification.

Since the method of the invention is based on the detection of a particular kind of DNA polymorphism, which occurs in DNA of any organism, the invention will be universally applicable. The methods of the present invention may be used to genotype ESPs in a wide variety of organisms from prokaryotic organisms, such as bacteria, through complex eukaryotic organisms, viruses, or any organism having a genome however simple or complex. The methods may also be used for the analysis of extrachromosomal DNA, the DNA found in certain cellular organelles, cDNA preparations, or DNA libraries, such as yeast artificial chromosome libraries and others. Furthermore, based on the large body of DNA sequence data at hand, it is predicted that the genomes of higher organisms carry several hundreds of thousands of such DNA polymorphism. Consequently, the new method is capable of diagnosing the immense number of genetic markers that are needed to unravel complex traits. The method is of tremendous value for high throughput genetic analysis in the emerging field of pharmacogenomics. Similarly, the method has great potential in the field of animal and plant breeding, where high resolution genetic analysis will be needed to identify the genes involved in quantitative agronomic traits.

Various aspects of the present invention are described in more detail below (*see* Detailed Description of the Invention). Variations in each of these aspects will be readily appreciated by one of ordinary skill in the art and one with the scope of the invention.

Brief Description of the Drawings

Figure 1 depicts the general concept of the Restricted Amplicon Assay. The vertical arrows indicate the positions of the ESPs. The open circles denote the probing enzyme sites that are present, while the closed circles denote the mutated sites. The first step involves cleavage of the test DNA with the probing endonuclease. The second step involves PCR amplification of DNA segments comprising the ESPs. The

small horizontal arrows denote the PCR primers flanking the ESPs. When cleavage occurs the DNA is cut between the PCR primers, preventing the subsequent amplification of the DNA segment comprising those ESPs. Only those DNA segments that were not cleaved are amplified. The final step comprises assaying the amplicons.

Figure 2: Diagrammed representation of format-I RAA. The vertical arrows indicate the positions of the ESPs, with the open and closed circles denoting the probing enzyme sites that are respectively present and absent. Step 1 represents the sampling enzyme cleavage step. The vertical dotted arrows indicate the positions of the sampling enzyme cleavage sites. Step 2 represents the adapter ligation step. The open lines represent the adapters ligated to the ends of the sampled restriction fragments. Step 3 represents the probing enzyme cleavage step and the small horizontal arrows denote the PCR primers matching the adapter sequences. Step 4 represents the PCR amplification step in which only the sample fragments that are not cleaved by the probing enzyme are amplified. The crossed circles represent the fragments that are not amplified.

Figure 3: Diagrammed representation of format-II RAA. The vertical arrows indicate the positions of the ESPs, with the open and closed circles denoting the probing enzyme sites that are respectively present and absent. Step 1 represents the probing enzyme cleavage step. The dotted boxes denote the DNA sequences flanking the ESP sites. Step 2 represents the PCR primer design. The small horizontal arrows denote the PCR primers flanking the ESPs Step 3 represents the PCR amplification step in which only the sample fragments that are not cleaved by the probing enzyme are amplified. The crossed circles represent the fragments that are not amplified.

Figure 4: Diagrammed representation of format-III RAA. The vertical arrows indicate the positions of the ESPs, with the open and closed circles denoting the probing enzyme sites that are respectively present and absent. Step 1 represents the sampling enzyme cleavage step. The vertical dotted arrows indicate the positions of the sampling enzyme cleavage sites. Step 2 represents the pre-amplification step in which the sampled fragments are amplified. Step 3 represents the probing enzyme cleavage step. Step 4 represents the PCR primer design. The small horizontal arrows denote the PCR primers flanking the ESPs. Step 5 represents the PCR amplification step in which

only the sample fragments that are not cleaved by the probing enzyme are amplified. The crossed circles represent the fragments that are not amplified.

Figure 5: Graphic representation of target fragments produced by cleavage with a hexacutter (full arrows) and a tetracutter (dotted arrows) restriction enzyme. Two types of fragments are produced: type I fragments (dotted lines) carrying two tetracutter ends and type II fragments (full lines) carrying one hexacutter end (represented by the arrowhead) and one tetracutter end. Upon PCR amplification only the type I fragments are amplified.

Figure 6: EcoRI-BfaI fragments from ecotypes Columbia (C) and Landsberg (L) obtained after selective amplification using EcoRI and BfaI AFLP primers with respectively 2 and 3 selective nucleotides. The fragment patterns were obtained respectively without probing enzyme (no enzyme) and after digestion with the MseI probing enzyme. It is noted that most of the larger fragments do not survive after MseI digestion, while the majority of the smaller fragments survive the treatment. The differences between the ecotypes Columbia (C) and Landsberg (L) observed after MseI digestion, marked by the arrows represent ESP carrying fragments. The differences found without MseI digestion, marked by the stars represent typical AFLP polymorphisms.

Figure 7: False color hybridization patterns obtained on the Arabidopsis micro-arrays. The layout of the Arabidopsis micro-array is as follows: the left panel contains the ESP fragment probes derived from Columbia (upper half) and Landsberg (lower half), while the right panel contains the control monomorphic probes with respectively the negative control fragments (-control) always carrying a probing endonuclease site and the positive control fragments (+control) carrying no probing endonuclease site. The upper part of the figure shows the hybridization patterns obtained with uncleaved sample DNA, while the lower part of the figure shows the hybridization patterns obtained with cleaved sample DNA. The false color code is as follows: green represents hybridization with the Cy3-labeled fragments, red represents hybridization with the Cy5-labeled fragments, yellow represents hybridization with both the Cy3-labeled and the Cy5-labeled fragments, and gray represents no hybridization. In this figure of a set of idealized results is presented. The hybridization

patterns with the uncleaved sample DNA shows that all probes detect sequences in both ecotypes, while the hybridization patterns with the cleaved sample DNA show that the ESP fragment probes detect only the sequences in the respective ecotypes from which the ESP fragments were isolated. In addition, fragments carrying no site for the probing enzyme, detect sequences in both ecotypes, while fragments that always carry a site for the probing enzyme do not show a hybridization signal.

Figure 8: False color hybridization patterns obtained on the corn micro-arrays. The layout of the corn micro-array is as follows: the left panel of probes contains random fragments derived from B73, while the right panel contains Mo17-fragments. The figure shows four hybridization patterns obtained with respectively uncleaved sample DNA, MseI-cleaved, Tsp509I-cleaved and AluI-cleaved cleaved sample DNA. The uncleaved sample DNA hybridization pattern shows probes that hybridize only to B73 (green), respectively Mo17 (red) fragments, which represent polymorphisms resulting from mutations in the sample enzyme recognition sites. The cross in the circle indicates that these probes are eliminated from the analysis. The cleaved sample DNA hybridization patterns show that the majority of the probes do not give a hybridization signal, indicating that their cognate fragments are cleaved by the probing enzyme. Most of the probes giving a signal hybridize to both sample DNAs. Those that hybridize to only one of the sample DNAs and that were eliminated represent fragments carrying ESPs. The white arrows denote the probes that were retained for further analysis.

Detailed Description of the Invention

The term "SNP" means Single Nucleotide Polymorphism, i.e. a polymorphism involving the mutation of a single base-pair.

The term "ESP" means Endonuclease Site Polymorphism, i.e. a polymorphism involving two alleles one of which is cleaved by an endonuclease reagent while the other exhibits (at least partial) resistance to cleavage by the same endonuclease under the same conditions.

The phrase "(restriction) endonuclease reagent" refers to a reagent that consists of one or more enzymes and that cleaves nucleic acids with a certain

specificity, i.e. cleavage involves recognition of a particular sequence or set of sequences in the target DNA. Endonuclease reagents include but are not limited to the common type II restriction enzymes.

The term "sampling endonuclease(s)" or "sampling enzyme(s)" refers to an endonuclease reagent used to derive sets of fragments from the sample DNA. The term "probing endonuclease(s)" or "probing enzyme(s)" refers to an endonuclease reagent used to probe the allelic state at specific ESP-sites.

The term "polymorphism" refers to the existence of two or more alleles at significant frequencies (≥1%) in the population; polymorphism at a single chromosomal location constitutes a genetic marker.

The term "micro-satellite (DNA)" refers to a small array (often less than 0.1 kb) of tandem repeats of a very simple sequence, often 1 to 4 base-pair. Variability at such a locus is the basis of many genetic markers.

The term "mutation" means a heritable alteration in the DNA sequence.

The term "allele" refers to one of several alternative sequence variants at a specific locus.

The term "genotype" is commonly known to mean (i) the genetic constitution of an individual, or (ii) the types of allele found at a locus in an individual.

The term "haplotype" refers to the genotype at a series of linked loci on a single chromosome.

The term "sample DNA" or "sample fragments" refers to the set of fragments or amplicons derived from the starting DNA by the RAA method.

The term "zygosity" refers to the homozygous or heterozygous state.

The term "homozygosity/homozygous" refers to the presence of identical alleles at a locus.

The term "heterozygosity/heterozygous" refers to the presence of different alleles at a locus.

The term "CpG" means a dinucleotide with a cytosine at the 5'-side and a guanine at the 3'-side. CpG is relatively rare in mammalian DNA because of the tendency for the cytosine to be methylated and subsequently mutate to thymine by deamination.

The term "ecotype" refers to a naturally occurring (plant) variety; race.

The term "bi-allelic" refers to a polymorphic locus characterized by two different alleles.

The terms "microarray" and "(DNA-)chip" refer to a multitude of spatially addressable nucleic acids that serve as probes. The microarray may be used in the form of a planar solid support, a bead, a sphere, or a polyhedron. Fabrication is done either by in situ combinatorial synthesis of oligonucleotides using photolithography, or by robotic spotting of off-chip prepared DNA onto a solid surface.

The methods of the present invention differs conceptually from previously described restriction enzyme-dependent assays (*supra*) that essentially detect a fragment length polymorphism. With the present method, starting DNA is restricted prior to the amplification reaction and, rather than analyzing the obtained amplification product, the presence or absence of amplification is measured to determine the allelic state at an ESP site. The treated DNA is preferably amplified by using a polymerase chain reaction and is preferably analyzed by means of hybridization against arrays of probe DNAs. With the present method, a sample-amplicon, and consequently a hybridization signal, is either present or virtually absent. This feature represents a major advantage in that it results in a more accurate distinction between variable nucleotides than is possible by differential hybridization to allele-specific oligonucleotides, and because it greatly facilitates the identification of a set of generally useful hybridization conditions. Also, the methods of the invention permit the use of both oligonucleotides as well as DNA fragments as probe DNAs. While hybridization to arrays allows the simultaneous analysis of a large number of ESPs, it should be clear that the amplification of sample DNA, treated with probe restriction endonuclease reagent, can be analyzed by any of a variety of methods well known in the art. In these methods, an ESP is identified either by the presence of a recognition site for the probe restriction endonuclease reagent (which will result in the failure of the sample DNA to amplify) or by the loss of a recognition site which will allow amplification of an otherwise unamplifiable sample DNA. Alternative methods include, but are not limited to, gel-electrophoretic analysis, and the TaqMan assay [Holland P. M. *et al.*, *Proc.*

Natl. Acad. Sci. **88**: 7276-7280 (1991); with the latter assay detection is done during rather than after the amplification reaction].

One of the advantages of the method of the invention is the ability to calibrate the measured signal against that obtained in a control experiment where digestion with the probe restriction endonuclease reagent is omitted. Comparison of the respective hybridization signals, following various corrections and normalization procedures, is essential for the genotyping of ESPs and the accurate determination of the zygosity. The cleaved and uncleaved material can, in principle, be hybridized separately but a preferred method consists of hybridizing a mixture of the differentially labeled samples to the same array. The present invention is exemplified by several specific formats described below.

(I) Format-I RAA: Choice of sampling and probing restriction endonuclease reagents. In one of its embodiments the present invention is directed to methods for detecting ESPs in a "restricted amplicon assay" (RAA) which comprises preparing concomitantly amplifiable restriction fragments from the starting DNA (sample DNA). When generating discrete sets of DNA fragments from genomic DNA, the following parameters are important: the average fragment size and the total number of fragments. The optimal fragment size for use in the methods (and materials) of the present invention is a trade off; the fragments must be sufficiently small for amplification with roughly equal efficiency (in general ≤500 base pairs) and large enough for having on average one cleavage site for the probing endonuclease reagent. In addition to average fragment size, the number of fragments determine the complexity of the sample DNA which is critical in view of the limitations of the detection sensitivity of micro-array hybridization. In general, the current state of the art of microarray hybridization is such that the number of sample fragments should not exceed 100,000. All of the above-mentioned requisites can be met by the appropriate choice of sampling and probing enzymes. A preferred method of the present invention to prepare sample DNAs (amplicons) involves the use of two different sampling enzymes, a rare cutter endonuclease (*e.g.*, hexacutter) combined with a frequent cutter endonuclease (*e.g.*, tetracutter), as described in EP 0 534 858 A1 which describes a method called AFLP and which is incorporated herein by reference. As can be seen

from Figure 5, the rare cutter enzyme produces large fragments that upon cleavage with the frequent cutter enzyme are cut into a number of smaller fragments. This dual cleavage generates two types of fragments: the majority having both ends produced by the frequent cutter (type I) and a minority of fragments having a rare cutter end and a frequent cutter end (type II). After ligating different adapters to each of the ends and using appropriate primers targeted to the ends of the fragments, only the type II fragments will be amplified efficiently (see Figure 5). The type I fragments amplify with greatly reduced efficiency presumably because the synthetic sequences at the two ends constitute an inverted repeat. In general the type II fragments will amplify synchronously using a single PCR primer pair that attaches to the ends of the fragments. The size limit is typically around 500 base pairs, but can be increased by using a different DNA polymerase and other reaction conditions. Thus, as outlined above the number of amplifiable fragments will be determined primarily by the choice of the rare cutter restriction enzyme. By approximation, this number equals two times the number of cleavage sites for the rare cutter. In a preferred embodiment, restriction enzymes recognizing 6 nucleotides (hexacutters) or more are used as rare cutters. The use of a frequent cutter recognizing 4 nucleotides (tetracutter) as second sampling enzyme results in the production of fragments in the optimal size range for co-amplification. As probe restriction endonuclease reagents, different tetracutter or pentacutter enzymes can be used. The probe restriction endonuclease reagent and the frequent cutter sampling enzyme should preferably be chosen such that the ratio of the cleavage frequencies of probing over sampling reagent is >0.5 and <3. This will ensure that a substantial fraction of the target fragments are cleaved once by the probing enzyme. It is noted that ESPs cannot be genotyped when the fragments are cleaved more than once by the probing enzyme. Also, it should be recognized that cleavage with the probe restriction endonuclease reagent results in a significant reduction (typically 2-4 fold) of the fragment complexity.

Alternative schemes - different from the one described above - that meet the requisites of sample complexity, average fragment size, and occurrence frequency of the probe reagent and that will perform equally well, will be readily apparent to one of ordinary skill in the art. Alternative schemes may include the use of pairs of

frequent cutters, followed by selective amplification (described in EP 0 534 858 A1), or the use of type IIS restriction enzymes. Type IIS restriction enzymes are characterized by an asymmetric recognition sequence. Most of these enzymes cleave at a defined distance to one side of the recognition site and generate single stranded overhangs that have different sequences. Ligation of adaptor sequences that are complementary to only one type of overhang allows the amplification of specific subsets of fragments [Kikuya Kato, *Nucleic Acids Res.* 23: 3685-3690 (1995)]. With this strategy the set of fragments obtained with the sampling enzymes can be broken up in a defined number of complementary and roughly equally complex subsets. Thus, with these enzymes it is possible to tune the complexity of the sample. The same strategy can be applied by making use of type II enzymes that have an interrupted palindromic recognition sequence.

Type of mutations detected by format-I RAA: In essence the method of the invention aims to detect mutations affecting the recognition sequences of the site-specific probe endonuclease reagents. When the probe enzyme cleaves a sample fragment, it is prevented from being amplified and as a consequence the fragment will not give a hybridization signal with its cognate probe. Mutations affecting the recognition sequence of the probe enzyme will allow amplification of the sample fragment and will restore the hybridization signal. It is recognized that mutations other than those affecting the probe enzyme recognition sites may affect the hybridization signals. In particular, mutations affecting the recognition sites of the sampling enzymes may also lead to a loss of hybridization signal. Consequently, the mere detection of a hybridization difference between two samples does not qualify the difference as being due to an ESP for the probing enzyme. For this one must also assay the two samples without probing enzyme cleavage; only those differences that are correlated with the cleavage by the probing enzyme qualify as genuine ESPs as defined according to the present invention. Therefore, a preferred embodiment of the methods of the present invention comprise the comparison of the hybridization signals obtained with and without cleavage of the same starting material by the probe endonuclease reagent. Preferably, the digested and undigested sample DNAs are differentially

labeled such that equivalent amounts of the material can be mixed and hybridized against the same array of probes. It is noted that a further advantage of measuring the relative hybridization signals obtained with digested and undigested sample DNAs, is that the signal given by the undigested sample DNA serves as an internal control for correcting variations in amplification and hybridization.

Identification and design of informative probes to detect ESP-harboring fragments. In a preferred embodiment of the present invention sample DNAs (amplicons) are hybridized to micro-arrays comprising a set of probe DNAs which are designed such that each probe will hybridize specifically to one sample DNA fragment. For each set of sample DNA fragments a specific set of probes are developed that will detect all the ESPs present in the set of sample DNAs. Since in most applications only a (minor) fraction of the sample DNAs will actually carry an ESP for a particular probing reagent, the set of probe DNAs will preferably consist of a subset of the sample DNA fragments that are informative in that they hybridize to ESP-harboring sample fragments. Preferably, the probes are highly specific for the ESP-carrying sample fragments, and do not cross-hybridize with other fragments in the sample. This feature is verified by testing the candidate probes in control hybridization assays. When developing or designing the probes care should be taken to avoid hybridization of the labeled primer used to amplify the sample fragments. When the probes correspond to a subset of the sample fragments, preferably an alternative set of adaptors should be used for their amplification.

The sections below describe different approaches that may be used to assemble sets of unique probe DNAs for fabricating the micro-arrays. Three alternative approaches are presented, and their choice is determined primarily by the degree of nucleotide sequence variation, and hence the ESP frequency, present in the species under study.

(1) Direct screening. When the ESP frequency is high, such that 10% or more of the sample fragments carry ESPs, a realistic approach for assembling ESP probes is to array individual sample fragments and test which of them detect an ESP in the test material under study. The advantage of this approach is that the same set of

fragments can be tested with different probe enzymes. After the screening one will retain only those probes that yield a clear-cut difference in hybridization between the different test DNAs. This approach is illustrated in Example 2.

(2) Gel-based screening. With genomic DNA exhibiting intermediate ESP frequencies (a few %), useful probes can be identified with a gel-based screening approach in which the ESPs are identified by comparing the patterns of sample fragments obtained from cleaved and uncleaved genomic DNA of various individuals. The polymorphic fragments can then be isolated from the gel and cloned or amplified. In a second phase, these probe-fragments are verified in a micro-array hybridization assay. This approach is illustrated in Example 1.

(3) Batch-wise hybridization selection method. Since both approaches described above are inefficient and labor intensive when the ESP frequency is low (<1%), it is advantageous to directly select or enrich ESP-carrying fragments. Such an approach is described in greater detail in Example 3.

The methods of the invention can be used with any type of micro-array: spotted ESP-carrying fragments, spotted oligonucleotides or oligonucleotides synthesized on solid supports using photolithography [Fodor S. P. A. *et al.*, *Science* **251**: 767-773 (1991)]. Oligonucleotide probes can easily be designed based on the nucleotide sequences of the ESP-carrying fragments. Also, the methods of the invention are not limited to the use of planar arrays containing spatially addressable probes. A person of skill in the artwill recognize that the methods may alos employ a multitude of identifiable solid phase particles (e.g. beads, spheres, and polyhedron), each carrying a different probe. Examples of such use are described by Fulton, R. [U.S. Patent No. 5,736,330] and Mandecki, W. [U.S. Patent No. 5,736,332].

(II) Format-II RAA General outline

The 'format-I RAA' - as described above - can be converted to a 'format-II assay' when sufficient sequence information of ESP-containing sample fragments becomes known. Format-II RAAs can also be designed on the basis of the known sequences of genomic regions that harbor an ESP and that are available through publicly accessible databases. The approach involves the targeted sampling of starting

material and consists of the design of dedicated primer pairs that flank the ESP sites. Like in format-I RAA, if the site is intact, the starting DNA will be cleaved and no PCR product will be generated. Only when the site is mutated will the amplicon be generated. In practice, multiple ESP-containing genomic regions are co-amplified after cleavage with the probing restriction endonuclease reagent. The ultimate sample DNA used in the hybridization reaction is composed of several such multiplex PCR reactions pooled together. The feasibility of this approach is evidenced by the recent paper of Wang *et al., Science* **280**: 1077-1082 (1998), incorporated herein by reference. The methods for format-II RAA described here are identical to the approach described by Wang *et al.*, in the way certain allelic regions are co-amplified, but fundamentally different in the way they are diagnosed. The present method takes advantage of the clear distinction between having or not having an amplicon depending upon the allelic state of the endonuclease target site. The Wang *et al.* approach in contrast relies on the detection of a hybridization difference as a result of a single nucleotide variation in the PCR product. This requires a much more elaborate and redundant hybridization assay.

Similar to format-I RAA, a preferred method consists of comparing the hybridization signals obtained with and without cleavage with the probe restriction endonuclease reagent. Preferably, the respective amplification reactions are differentially labeled such that the resulting amplicons can be mixed and hybridized against the same array of probes.

Preferred methods of the format-II RAA are those wherein - of each PCR primer pair - that primer that remained unlabeled is used as hybridization probe for the corresponding amplicon. This ensures that the excess unincorporated labeled primer as well as the primer extension products obtained with this primer cannot anneal to the arrayed probe. Also, the unlabeled PCR primer is complementary to the labeled strand of the amplicon.

Furthermore, the format-II RAA method provides a means to monitor mutations in specific genes or loci in addition to scanning the entire genome. Indeed, sets of PCR primers that target ESPs in a specific gene or chromosome region can be assembled.

An RAA assay with positive detection of both alleles:. It is recognized that the 'present/absent-score' of the RAA assay cannot (always) distinguish between different mutations that can affect cleavage by the probe restriction endonuclease reagent. In practice, an ESP should not be assayed when available evidence indicates the existence of two or more such mutations at significant frequencies in the population.

In a preferred embodiment the present invention is directed to the detection of SNPs that result in the simultaneous loss and gain of a restriction enzyme recognition site, *i.e.* both alleles are associated with a different recognition site. HgaI (GACGC) and SfaNI (GATGC) are an example of such reciprocal sites. Use of both probing endonuclease reagents in side-by-side experiments excludes alternative alleles and results in easy determination of the zygosity (refer to Example 4).

Multi-allelic haplotyping:. A single ESP represents a bi-allelic marker, which is less informative than a variable micro-satellite, which has multiple alleles. It is possible however to compensate for the lower information content by identifying several ESPs on a specific chromosomal region. Format-II RAA lends itself readily to such an approach and involves the design of a primer pair that encompasses a region with a single site for the various selected probe endonuclease reagents. It should be recognized as one of the advantages of the present method that multiple ESPs on a sample amplicon can be interrogated with a single probe. Furthermore, use of the probing enzymes, either separately or in various combinations, in parallel experiments allows the construction of the haplotypes for the ESPs under study. In general, the statistical associations between traits and specific chromosome regions may be more apparent when haplotypes rather than individual markers are used.

(III) Format-III RAA:

In a general sense, the format-III RAA represents a method of choice for very high-density SNP genotyping because it provides a means to overcome the intrinsic limitations of both the format-I RAA and the format-II RAA. This is essentially achieved by performing a stepwise amplification involving a pre-

amplification of sample fragments followed by amplification using multiplexed specific primers. The principal advantage of the pre-amplification step is to reduce the complexity of the starting DNA, and thus to provide a more favorable starting point for performing multiplex PCR reactions. It is noted that this improvement is generally applicable to any multiplex PCR reaction, and is not limited to the methods of the present invention. Such an approach can also be used when for example SNPs are genotyped using the methods described by Wang *et al.*

The principal limitation of the format-I RAA lies in the complexity of the sample DNA that is hybridized to the microarray. Because the second round of amplification in format-III yields only very small amplicons, which are all informative, there is no longer a limitation in number of sample fragments that are interrogated. In fact the entire genome may be sampled in a series of parallel pre-amplification reactions and the amplicons generated in the different multiplex PCR reaction can then be pooled together and hybridized to the microarray.

Likewise, the format-III RAA represents preferred methods of format-II RAA, especially when the ESPs under study are located on fragments generated by one set of sampling endonuclease reagents. Such stepwise amplification comprises the co-amplification of sample fragments with a single pair of primers, followed by the selective amplification of sets of specific ESP-containing regions (see Figure 5). The principal advantage of the format-III RAA over format-II RAA is that the initial amplification of the sampling fragments - representing only a fraction of the total genome - lowers the amount of starting material required to interrogate a very large numbers of ESPs. Also, the approach will facilitate the multiplex amplification of the ESP-specific amplicons and, consequently, yield a more robust assay.

One preferred embodiment of the format-III RAA is its use to genotype large numbers of ESPs identified through the use of the format-I RAA. Indeed, format-I RAA offers a rapid means to discover large numbers of ESPs in any biological species where no large body of sequence information is or will be available. Format-I RAA enables one to discover many sets of ESPs for a number of different probing enzymes. Using the format-I RAA, each set of ESPs must be assayed on a different microarray, because otherwise signals for the same sample fragment will overlap with

one another, and thus preclude the proper ESP genotype to be determined. Using the format-III RAA, the ESPs identified with different probing enzymes are now assayed together on one single microarray, without overlap between the different ESPs. The reason is that the overlap in the format-I RAA is caused by the non-informative sample fragments that are always co-amplified with the ESP fragments. These are eliminated from the mixture by the specific PCR amplification. This embodiment is illustrated in Examples 2 and 3.

Another preferred embodiment of the format-III RAA is its use to genotype large numbers of SNPs identified in high-throughput sequencing of genomic DNA from different individuals from a given species. Given the generally recognized importance of SNPs for the development of high-resolution genotyping methods, sequenced SNPs can be expected to accumulate in large numbers in publicly available databases in the near future. In particular, in the field of human genetic analysis, SNPs will be discovered at a rapidly increasing rate through the massive genome sequencing programs now in progress. A similar evolution may be anticipated for many other species. Hence we decided to perform an *in silico* analysis of known human SNPs to further investigate the potential of the invention. More particularly we have analyzed the 3,358 SNP sequences present in the SNP database of the Whitehead Institute [Wang *et al.*, *Science* **280**: 1077-1082 (1998)]. We have determined how many of these SNPs represent an ESP for each of 34 known palindromic and non-palindromic tetra- and penta-nucleotide restriction recognition sequences. When extrapolating this number to the total number of ESPs in the human genome - assuming a grand total of 3 million ESPs - it appears that the number of detectable ESPs per probing restriction enzyme is in the range of 25.000 to 150.000. A cumulative analysis reveals that 53% of the SNPs affect at least one of the 34 restriction sites; a total of 28% affect the recognition site for one of the available tetracutter enzymes. The principal conclusion from this analysis is that many of the considered enzymes - used as probing enzymes according to the methods of the present invention - will interrogate sufficient SNPs to be able to built a high-density map of the human genome. It should also be noted that the use of multiple probing enzymes is easily accommodated in the targeted assay because the sample has to be subdivided anyway over a number of parallel multiplex PCR

reactions. This embodiment is illustrated in Example 4.

It is noted that the format-III RAA may be performed according to different procedures. One such procedure is diagrammed in Figure 5, in which the test DNA is first sampled using a sampling endonuclease reagent, pre-amplified and then treated with the probing endonuclease reagent. Variations on this procedure are readily recognized by those skilled in the art and include for example, concomitant treatment of the test DNA with both the sampling and the probing endonuclease reagents and the preparation of sampled DNA fragments using arbitrary PCR priming methods [Williams *et al.*, *Nucleic Acids Res.* **18**: 6531-6535 (1990)]. Note that in case the treatment with the probing endonuclease reagent is performed prior to the pre-amplification, the subsequent amplification can be performed with any pair of PCR primers directed against the ESP carrying fragments, and thus overcoming the limitation of using PCR primers flanking the ESPs.

Table I. Analysis of 3,358 SNPs in the Whitehead SNP database. The table lists the number of SNPs that represent an ESP for various probing enzymes. The last column shows the estimated number of ESPs for each enzyme in the entire human genome (refer to text for details).

Type of probing reagent	enzyme	site	ESPs	total number of ESPs
Tetracutter enzymes	Tsp509I	AATT	122	109.000
	MaeII	ACGT	106	95.000
	AluI	AGCT	98	88.000
	NlaIII	CATG	158	141.000
	MspI	CCGG	77	69.000
	BstUI	CGCG	27	24.000
	BfaI	CTAG	67	60.000
	Sau3A	GATC	58	52.000
	HinPI	GCGC	49	44.000
	HaeIII	GGCC	52	46.000
	Csp6I	GTAC	71	63.000
	TaqI	TCGA	50	45.000
	MseI	TTAA	109	97.000
Pentacutter enzymes	Tsp4CI	AGNCT	114	102.000
	BssKI	CCNGG	79	71.000
	DdeI	CTNAG	122	109.000
	HinfI	GANTC	77	69.000
	Fnu4HI	GCNGC	71	63.000
	Sau96I	GGNCC	64	57.000
	MaeIII	GTNAC	70	63.000
Non-palindromic enzymes	AciI	CCGC	111	99.000
	MnlI	CCTC	175	156.000
	BbvI	GCAGC	65	58.000
	BsmAI	GTCTC	67	60.000
	BsmFI	GGGAC	39	35.000
	FokI	GGATG	66	59.000
	HgaI	GACGC	31	28.000
	PleI	GAGTC	39	35.000
	SfaNI	GCATC	51	46.000
	AlwI	GGATC	37	33.000
	BsrI	ACTGG	76	68.000
	HphI	GGTGA	69	62.000
	MboII	GAAGA	85	76.000
	TspRI	CAGTG	94	84.000

The following illustrative examples were chosen to represent the spectrum of genomic complexities and the spectrum of degrees of genetic variation which are susceptible to analysis using the methods of the present invention:

Example 1 describes analysis of Arabidopsis (low genomic complexity, low genetic variation).

Example 2 describes genetic analysis of corn (high genomic complexity, high genetic variation).

Examples 3 and 4 describe genetic analysis in humans (high genomic complexity, low genetic variation).

Numbers given in the examples, and that relate to the occurrence frequency of certain restriction sites as well as the average size of the generated fragments are in part based on computer simulations using publicly available DNA sequences.

Example 1

Genetic Analysis in Arabidopsis

In this example, a fragment analysis-based approach is used to generate a set of genomic fragments carrying ESPs between the Arabidopsis ecotypes Landsberg and Columbia, which are commonly used for genetic studies in the model organism. Arabidopsis is an example of a low complexity genome (size ~120 Mb), and the two ecotypes exhibit a moderate level of genetic variability. Previous studies have revealed that the average nucleotide sequence variation between the two ecotypes is in the order 1 polymorphism in 150 nucleotides. Consequently, the fraction of fragments expected to carry an ESP for tetranucleotide recognizing restriction enzymes is expected to be in the range of 2.5% (1:40). With such a low frequency, it is helpful to use a selection procedure to isolate the rare fragments containing ESPs.

In essence the procedure described in this example comprises the following steps:

4) Identification of a set of about 200 genomic fragments carrying Landsberg/Columbia ESPs using a gel-electrophoretic approach.
5) Isolation and characterization of the ESP carrying DNA fragments (ESP fragments).
6) Generation of micro-arrays with the ESP fragments
7) Confirmation of the ESPs by hybridization.

Step 1. Identification of ESP fragments.

Sampling enzymes. In the present example EcoRI, a restriction enzyme recognizing 6 nucleotides (hexacutter), in combination with BfaI, a restriction enzyme recognizing 4 nucleotides (tetracutter), are chosen as sampling enzymes. From the random frequency of occurrence of 6 nucleotide sequences (every 4,000 bases), the number of sites for hexacutter restriction enzymes in this genome is predicted to be in the range of 30,000. In addition to cleavage with a hexacutter, the genomic DNA is also cut with a tetracutter so as to generate PCR amplifiable fragments of an average size of a few hundred base pairs. Cleavage with the two enzymes gives rise to two types of fragments: a majority of fragments resulting from cleavage by the tetracutter

enzyme alone and a smaller set of fragments produced by the two enzymes (see Figure 5). Since the majority of the hexacutter fragments will give rise to two fragments having a hexacutter end and a tetracutter end (*see* Figure 5), this procedure will yield a mixture of about 60,000 fragments of this type. Upon amplification using the procedure described below only the fragments carrying a tetracutter end and a hexacutter end are amplified efficiently (Figure 5).

Probing enzymes. As probing enzymes many different tetracutter enzymes can be used. Ideally, the probing enzyme cleaves most of the sample fragments once. Because plant DNA has a high AT content, the preferred tetracutters are those that have an AT bias in their recognition sequence. In general, the choice of an optimal tetracutter may be determined by particular features of the genome being analyzed (*e.g.*, AT and GC content). In the present example, MseI (recognition site = TTAA) was chosen. Tsp509I (recognition site = AATT) is an alternative. It is also conceivable to use mixtures of two or more tetracutter enzymes. The EcoRI-BfaI sample/target fragments that are cleaved and not cleaved with the MseI probing enzyme are referred to as cleaved and uncleaved sample/target DNA, respectively.

Screening for ESP carrying fragments. To detect ESP fragments, subsets of uncleaved and cleaved EcoRI-BfaI sample fragments from both ecotypes are amplified and the amplicons are compared following gel-electrophoretic fractionation. Subsets of the EcoRI-BfaI sample fragments are selectively amplified as described [Vos, P. *et al.*, *Nucleic Acids Res.* 23: 4407-4414 (1995); Zabeau, M. and Vos, P., European Patent Application EP 0534858 (1993) both of which are incorporated herein by reference]. Given the complexity of the sample (~50,000 fragments), the selective amplifications are performed with EcoRI and BfaI primers having two and three selective nucleotides, respectively. This equals 1024 (16 x 64) different selective amplification reactions.

The experimental procedure described by Vos P. *et al.* is followed except that the template fragments are incubated at 65°C during 10 minutes to heat-inactivate the T4 ligase enzyme, and, when applicable, digested with the probing enzyme prior to amplification. The structures of the EcoRI and BfaI adaptors are as follows [*see, e.g.*, Vos, P. *et al.*, ***supra***]:

5'-CTCGTAGACTGCGTACC (SEQ ID NO: 1)
CATCTGACGCATGGTTAA-5' (SEQ ID NO: 2)

5'-GACGATGAGTCCTGAG (SEQ ID NO: 3)
TACTCAGGACTCAT-5' (SEQ ID NO: 4)

The EcoRI (radiolabeled by 5'-phosphorylation) and BfaI primers, having two and three selective nucleotides, respectively, have the following sequences (where N represents A, C, G, or T):

5'-GACTGCGTACCAATTCNN (SEQ ID NO: 5)

5'-GATGAGTCCTGAGTAGNNN (SEQ ID NO: 6)

Using these reagents, most of the obtainable target fragments contain a cleavage site for the probing enzyme and, consequently, will not be amplified when the target DNA is cleaved. Most of the fragments that survive the treatment with the probing enzyme occur in both ecotypes, and thus carry no ESP. Occasionally fragments are found that appear in both ecotypes when the target DNA is not digested and that are present in only one of the two ecotypes after digestion. These represent true ESPs for the probing enzyme. In addition, fragments will also be found that show typical AFLP-polymorphism between the two ecotypes [Vos, P. *et al.*, *Nucleic Acids Res.* **23**: 4407-4414 (1995)]. Such polymorphisms are apparent in the fragment patterns obtainable with the undigested sample DNAs. A typical result is shown in Figure 6 in which the electrophoretic patterns are shown of selectively amplified EcoRI-BfaI fragments from the Ecotypes Columbia and Landsberg obtained without and with digestion with the MseI probing enzyme.

Systematic comparison of the patterns of ecotypes Columbia and Landsberg before and after digestion, allows the identification of EcoRI-BfaI sample amplicons that carry an ESP for the probing enzyme. Using MseI as probing enzyme, it is estimated that a total of ~200 polymorphic fragments which are present in only one of the ecotypes can be identified.

Step 2. Isolation and characterization of ESP fragments.

Each of the ESP polymorphic fragments is eluted from the gel-matrix, re-amplified and cloned into a suitable plasmid vector (e.g. TA cloning system; Invitrogen, Carlsbad, CA, U.S.A.). In each case, two clones are selected for sequence determination. Most duplicate clones will yield the same sequence. Duplicate clones that gave different sequences were not retained for further work. Since the nucleotide sequence of over one third of the Arabidopsis genome is available in the public databases (*e.g.*, Genbank), the chromosomal location of one third of the ESP fragments can be determined by matching the fragment sequences to the genomic sequence. Furthermore since the genomic sequence is derived from ecotype Columbia, we expect a perfect match with the fragment sequences isolated from the same ecotype. The sequences of the fragments isolated from ecotype Landsberg will reveal single nucleotide differences, amongst which the potential restriction site mutations, affecting the MseI recognition sites, should be apparent.

In addition to the ESP polymorphic fragments, a number of non-polymorphic control fragments are processed in the same way. Two types of such control monomorphic fragments are isolated: fragments that do not carry a site for the probing enzyme and fragments that carry a site for the probing enzyme in both ecotypes. These fragments will serve the purpose of verifying the hybridization on the micro-arrays.

Step 3. Fabrication of ESP micro-arrays.

Micro-arrays of amplified fragments. The insert DNAs from the sequence verified clones are amplified, e.g. with the use of non-selective EcoRI and BfaI primers. PCR products are verified by agarose gel electrophoresis and retained if a single product of the correct mobility was present. Following ethanol precipitation, the resuspended PCR products are arrayed at high density on standard glass slides (25 x 76 mm) using either the Multigrid robotic spotter (GeneMachines™, Genomic Instrumentation Services Inc., Menlo Park, CA, U.S.A.) or the BioChip Arrayer™ (Packard Instrument Company, Meriden, CT, U.S.A.). The DNAs are spotted in a logical order with respect to the ecotype from which the fragments were isolated (upper

and lower panel) as shown in Figure 7. In addition, a set of DNAs from monomorphic control fragments was spotted next to the ESP fragment DNAs (right panel in Figure 7).

Micro-arrays of oligonucleotides. Based on the nucleotide sequences of the ESP fragments, oligonucleotides can be designed that can serve as hybridization probes to specifically detect each amplified sample fragment. The oligonucleotide probe should preferably match with a sequence that is located to one side of the ESP, opposite the side where the sequence targeted by the labeled primer is located. In this way the background is minimized because the linear amplification products generated by the labeled primer following digestion with the probing enzyme are not detected. The ESP fragment specific oligonucleotides are spotted in a micro-array format in exactly the same way as the amplified ESP fragments.

Step 4. Micro-array-based detection of ESPs.

Preparation of the sample DNAs. For each ecotype, sample DNA is prepared in two different ways. Genomic DNA, digested with the sampling restriction enzymes EcoRI and BfaI, was amplified either as such or after cleavage with the probing enzyme MseI. The amplification reactions are performed with a fluorescently labeled EcoRI primer and an unlabeled BfaI primer, both without selective nucleotides. The EcoRI primer is labeled by incorporation of Cy3(green)- and Cy5(red)-amidites during primer synthesis (Amersham Pharmacia Biotech, Uppsala, Sweden). For both Columbia and Landsberg, the cleaved sample was amplified with a Cy3-primer while the uncleaved fragments were amplified with a Cy5-labeled EcoRI primer. In addition, the Landsberg digested material was also amplified with a Cy5-labeled EcoRI PCR primer. Three different hybridization solutions are then prepared by mixing equal amounts (i.e. equal volumes) of the Cy3- and Cy5-labeled amplification reactions: one from the Columbia cleaved and uncleaved samples, a second from the Landsberg cleaved and uncleaved samples, and a third by mixing the differentially labeled cleaved samples of both ecotypes.

In case arrays of PCR products, rather than oligonucleotides, are used

as probes (refer to step 3), the co-amplification of the EcoRI-BfaI sample fragments is preferably accomplished with a pair of adaptors that differs from those attached to the arrayed probes. The alternative EcoRI and BfaI adaptors have the following structure:

5'-GAGCATCTGACGCATCC (SEQ ID NO: 26)
GTAGACTGCGTAGGTTAA-5' (SEQ ID NO: 27)

5'-CTGCTACTCAGGACTG (SEQ ID NO: 13)
ATGAGTCCTGACAT-5' (SEQ ID NO: 14)

The cognate non-selective EcoRI and BfaI primers have the following sequences:

5'-CTGACGCATCCAATTC (SEQ ID NO: 28)

5'-CTACTCAGGACTGTAG (SEQ ID NO: 16)

Micro-array hybridization. Each of the hybridization solutions is allowed to hybridize to the arrayed probes using protocols well known in the art. The experimental conditions depend primarily on the nature of the probes, PCR-amplified fragments versus oligonucleotides. Both types of experiments are amply described in literature: Wodicka, L. *et al.*, *Nature Biotechnol.* **15**: 1359-1367 (1997); Lockhart, D. J. *et al.*, *Nature Biotechnol.* **14**: 1675-1680 (1996); DeRisi, J. L. *et al.*, *Science* **278**: 680-686 (1997); Shalon, D. *et al.*, *Genome Res.* **6**: 639-645 (1996); Piétu, G. *et al.*, *Genome Res.* **6**: 492-503 (1196); Chee, M. *et al.*, *Science* **274**: 610-614 (1996); Wang D.G. *et al.*, *Science* **280**: 1077-1082 (1998); Winzeler E. A. *et al.*, *Science* **281**: 1194-1197 (1998), all of which are incorporated herein by reference.

A laser scanning system (ScanArray 3000; General Scanning Inc., Watertown, MA, U.S.A.) is used to detect the two-color fluorescence hybridization signals from the micro-arrays at a resolution of 10 micron per pixel. A separate scan is carried out for each of the two fluorophores used. Scanning parameters and laser power settings are adjusted to normalize the signal in the two channels (channel-1/Cy3; channel-2/Cy5). The obtained digital images were analyzed using the ImaGene™ image

analysis software (BioDiscovery Inc., Los Angeles, CA, U.S.A.). The extracted quantitative data are transferred to a spreadsheet for further analysis.

The present hybridization experiment is essentially set up as a confirmation of the gel-electrophoretic data (refer to step 1), and has, therefore, a predictable outcome. In addition, a number of control probes are included on the biochip that detect monomorphic EcoRI-BfaI Arabidopsis fragments (i.e., fragments on which a site for the probing enzyme is either present or absent in both ecotypes). The results from these control probes allow correction for background and optical cross-talk between the two channels, as well as calibration of the red and green hybridization signals. It is anticipated that the vast majority of the processed data are unambiguous with respect to the allelic state of a sample fragment and in agreement with the gel-electrophoretic analysis. Figure 7 shows a false-color representation of the idealized results of the present experiment using a fictitious array of probes. It cannot be excluded that certain hybridization results are not in agreement with the gel-electrophoretic assay and/or that certain probes do not allow unambiguous determination of the allelic state of the cognate sample fragment. Such probes should be excluded from the micro-arrays that are used to genotype experimental Arabidopsis samples, other than the Columbia and Landsberg controls used in the present illustrative example.

In routine genotyping experiments, either one of the hybridization schemes outlined above can be used. Determination of the allelic state can be done by comparing the hybridization signals obtained with and without cleavage of the starting DNA with the probe reagent. Alternatively, allele-calling could be based on a comparison of the signals obtained with the test-sample and an appropriate control (e.g. Columbia or Landsberg DNA), both cleaved with the probe endonuclease reagent. The samples that need to be compared can, in principle, be hybridized separately but a preferred method consists of hybridizing a mixture of differentially labeled samples to the same array.

Example 2

Genetic Analysis in Corn

In this example, the utility of the method of the invention for marker assisted selection applications in plant and animal breeding is illustrated. Corn has been chosen because it is a typical representative of crop species having a complex genome. The large size of the genome (2,400 Mb), the frequent occurrence of repetitive DNA sequences and the high degree of genetic variation, all constitute technical challenges. In this example, an approach based on the generation of a set of genomic fragments carrying ESPs from two well-known inbred lines of corn, B73 and Mo17 from which many of the corn elite lines are derived is used. Another reason for choosing these lines is that a well-studied recombinant inbred population derived from these lines is available. This population can be used to map the set of ESPs. The genetic map of ESP markers will prove to be an effective tool for genetic selection in corn breeding. It is evident, however, that a broader survey of the corn germplasm with a total of 10 to 20 lines will give a large number of additional ESPs (possibly 2 or 3 times as many) and will eventually result in a higher-resolution genetic map.

The ESP-harboring fragments could very well be identified by the gel-electrophoretic approach described for Arabidopsis (Example 1). However, an alternative strategy may be used given that the corn germplasm, like many crop species, exhibits a high degree of genetic variation. Indeed, based on previous studies, the average nucleotide sequence variation in the corn germplasm is estimated to be in the order of 1 difference in 15 to 30 nucleotides. This corresponds to a frequency in ESPs in the recognition sites of tetracutter restriction enzymes of 1 in 4. At this frequency it becomes feasible to directly examine arrays of random B73/Mo17-fragments for the presence of ESPs using the present RAA method without prior screening or selection. The strategy also lends itself readily to screening with several different probing enzymes.

In the present example, two different approaches for assaying ESPs are used. The first method (format-I RAA) is similar to the one described in Example 1, and detects ESPs in fragments sampled with a pair of restriction enzymes. In the second method (format-III RAA) individual ESPs are selectively amplified from the

sampled fragments with dedicated primer sets. The principal advantage of the latter approach is that ESPs detected with several different probing enzymes can be assayed simultaneously, and that multiplex amplification of ESP-specific PCR products is made considerably more robust.

In essence the procedure described in this example comprises the following steps:

8. Identification of a set of candidate ESP fragments from the inbred lines B73 and Mo17
9. Development of a corn ESP micro-array
10. Genetic mapping of a B73/Mo17 recombinant inbred population and of segregating populations

Step 1. Identification of candidate ESP fragments

Cloning of a set of sample fragments. To clone a set of random fragments from the inbred lines B73 and Mo17, the enzyme combination PstI and BfaI is used. The hexanucleotide-recognizing enzyme PstI was chosen because of the large size of the corn genome. It is estimated that this enzyme has around 30,000 sites in the corn genome. The second tetracutter-enzyme, BfaI, is expected to cleave in the majority of the cases on both sides of the PstI sites. The double digestion will therefore generate about 60,000 sample fragments with an average size of 400-500 base pairs.

Following double digestion of the genomic DNA, PstI- and BfaI-adaptors were ligated to the fragment ends and the material amplified with non-selective PstI and BfaI primers. The structures of the PstI- and BfaI-adaptors are based on those described by Vos P. *et al.*, *Nucleic Acids Res.* 23: 4407-4414 (1995):

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5'-CTCGTAGACTGCGTACATGCA (SEQ ID NO: 7)
   3'-CATCTGACGCATGT (SEQ ID NO: 8)

5'-GACGATGAGTCCTGAG (SEQ ID NO: 3)
    3'-TACTCAGGACTCAT (SEQ ID NO: 4)
```

The corresponding PstI and BfaI non-selective primers have the following sequences:

5'-GACTGCGTACATGCAG (SEQ ID NO: 9)

5'-GATGAGTCCTGAGTAG (SEQ ID NO: 10)

The amplification step enriches the PstI-BfaI fragments over the large excess of BfaI-BfaI fragments. After amplification the fragments are fractionated on an agarose gel to eliminate the fragments smaller than 100 base pair, and cloned in an appropriate vector (e.g. TA cloning system; Invitrogen, Carlsbad, CA, U.S.A.).

Preparation of spotted micro-arrays with the cloned sample DNA fragments. The insert DNAs, from the two libraries of cloned PstI-BfaI sample fragments (obtained from the B73 and Mo17 inbred lines), are amplified from the clones using the non-selective PstI and BfaI primers. Following purification and concentration, the amplicons are arrayed as described in Example 1. A total of 20,000 (i.e. 10,000 from each library) candidate probe DNAs are spotted.

Micro-array hybridization and selection of candidate ESP-fragments. From genomic DNA of the inbred lines B73 and Mo17 four different sets of PstI/BfaI-digested amplified DNA are prepared. An alternative pair of adaptors and non-selective amplification primers are used for this:

5'-GAGCATCTGACGCATGTTGCA (SEQ ID NO: 11)
3'-GTAGACTGCGTACA (SEQ ID NO: 12)

5'-CTGCTACTCAGGACTG (SEQ ID NO: 13)
3'-ATGAGTCCTGACAT (SEQ ID NO: 14)

5'-CTGACGCATGTTGCAG (SEQ ID NO: 15)

5'-CTACTCAGGACTGTAG (SEQ ID NO: 16)

The sample fragments are amplified either as such or after digestion with one of three alternative probing enzymes, MseI, Tsp509I and AluI. As probing enzymes many different tetracutter or pentacutter enzymes can be used. Because plant

DNA has a high AT content, the preferred enzymes are those that have an AT bias in their recognition sequence. Alternatively, mixtures of two or more tetracutter or pentacutter enzymes can be used.

For each of the B73 samples, a Cy3(green)-labeled PstI primer is used, whereas the Mo17-derived fragments are amplified with a Cy5(red)-labeled PstI primer (refer to Example 1). Different hybridization solutions are then prepared by mixing equal amounts of the uncleaved, MseI-cleaved, Tsp509I-cleaved, and AluI-cleaved samples of both inbred lines. Each of the 4 mixes is allowed to hybridize to the micro-arrays. Analysis of the scanned images involved normalization using the multitude of probes on the arrays that detect monomorphic fragments. Figure 8 shows a false-color representation of the idealized results of the present experiment using a fictitious array of probes.

Analysis reveals that candidate ESP fragments are readily identified by scoring the probes that hybridize with only one of the two inbred line sample DNAs after cleavage with the probe enzyme (Figure 8). The quantitative analysis allows us the use of an unambiguous cut-off threshold of 10-fold difference in the normalized signal intensities for scoring ESPs. It should be pointed out that the assay identifies both bona fide ESPs and polymorphisms in the sampling enzyme sites. Most of the latter polymorphisms result in a marked hybridization difference with the sample DNAs not cleaved with the probe enzyme (see Figure 8). Analysis of 180 probes reveals that roughly 6% of the sample fragments carry ESPs for MseI, Tsp509I, or AluI, in accordance with the expected ESP mutation frequency. The analysis of 20,000 cloned probe fragments is thus expected to yield a total of 1,200 fragments carrying ESPs for the three probe enzymes tested. By using additional tetracutter and pentacutter enzymes (see Table I), the fraction of ESP carrying fragments may be as high as 25%, amounting to 5,000 ESPs.

Of all probes that exhibit a differential hybridization with the cleaved sample DNAs, only those in which the recognition site for the probing enzyme is present were retained for development of a corn micro-array. Sequence determination of these probe-fragments reveals the position of the recognition site for the probe enzyme. Thus, we retained only those probes that failed to give a signal with the

cleaved sample DNA from the same inbred line from which they were isolated. Such probes exhibit the hybridization pattern shown in the Table here below and are marked with an arrow in Figure 8.

	B73/Mo17 (Cy3/Cy5) normalized hybridization signal	
	Undigested	MseI/Tsp509I/AluI-digested
B73-probes	~1	< 0.1
Mo17-probes	~1	> 10

Step 2. Development of a corn ESP micro-array

Sequencing of the candidate ESPs and design of marker specific primers. Clones corresponding to the probes that yield the desired hybridization pattern (Figure 8) are sequenced. The majority of the insert DNAs derived from these clones contain a single recognition site for the probing enzyme. For each unique candidate ESP, two specific PCR primers, flanking the restriction site, are designed.

In addition, the sequence of a limited set of probes that yielded invariant hybridization signals is also determined. PCR primers targeting these monomorphic sequences are included as references; they are used to calibrate the hybridization signals.

Validation of the candidate ESPs and fabrication of corn micro-arrays. The candidate ESPs, identified under step 1, are subjected to a confirmatory experiment using the format-III approach. First, four pre-amplification reactions are performed with a single primer pair and using the PstI-BfaI fragments, undigested or digested with either one of the three probing enzymes, as template material. These amplification reactions reduce the complexity of the DNA under study by more than two orders of magnitude while at the same time generating a large enough amount of material for the subsequent multiplex marker-specific PCRs. The pre-amplifications are then used for the PCR rescue of each of the characterized candidate ESPs using dedicated primer couples [refer to Wang, D. G. *et al.*, *Science* **280**:1077-1082 (1998)]. Particular sets of the ESP-specific primers that amplify the same type of ESP (i.e. ESPs for one particular probing enzyme) are combined in a single reaction, together

with the appropriate pre-amplification material as template. One of the ESP-specific primers is either Cy3- or Cy5-labeled; the other remained unlabeled. The Cy3-primers are used for the multiplex amplification of the DNA that had previously been digested with a probing enzyme, whereas the Cy5-primers are used with undigested control DNA. The PCR products from the various multiplex reactions performed on both digested and undigested DNA were pooled together to obtain a single hybridization mixture per starting DNA. The B73 and Mo17 derived material was analyzed in parallel experiments. The set of ESP-specific unlabeled PCR primers served as hybridization probes and was arrayed in the same way as amplification products. Conditions used are similar to those previously described for hybridization against oligonucleotide probes and are readily determined by one of ordinary skill in the art.

Direct comparison of the normalized Cy3 and Cy5 hybridization signals allows determination of the allelic state of the endonuclease target site in B73 versus Mo17. Primer pairs that do not allow unambiguous allele calling or that do not confirm the candidate ESPs identified with PstI-BfaI sampling (refer to step 1), are not retained for further work.

Step 3. Genetic analysis of a B73/Mo17 recombinant inbred population and of segregating populations

Genetic analysis of a B73/Mo17 inbred population. A collection of recombinant inbred lines derived from a cross between B73 and Mo17 is publicly available and provides a most useful set of lines for verifying and mapping the collection of ESP markers. The advantage of recombinant inbred lines over segregating populations is that each inbred line contains a different set of homozygous chromosome segments derived from either parent line. Consequently each ESP will be scored as either present or absent. Preparation of the sample DNAs and hybridization against the arrayed probes are performed as described under step 2. The experiment will, in the first place, allow the testing of selected ESPs in over 100 measurements; the results will result in the development of a second generation system that will only detect the most consistent ESPs. In addition, the linkage analysis of the segregation data will allow the construction of a fine genetic map of the markers. Finally, based on the

mapping data, an ordered ESP micro-array is developed for corn.

Genetic analysis of segregating populations. While isolated from two inbred lines, it is anticipated that the above-mentioned ordered ESP micro-arrays will detect sufficient genetic polymorphism in other corn lines to be useful for marker assisted selection. To demonstrate the applicability, one could either chose a segregating F2 population or a back-cross population. Sample preparations and hybridizations are again performed as described under step 2. In this experiment, the ESP markers must be scored quantitatively so as to differentiate between heterozygosity and homozygosity. Because only the most consistent markers are retained, a two-fold difference in signal intensity is easily monitored. The approach used consists of normalizing the hybridization signal intensities and then applying a mixture model analysis on the normalized data. This statistical approach consists of determining whether the relative signal intensities can be grouped into three discrete classes, corresponding to respectively homozygous present, heterozygous and homozygous absent. ESP markers that do not fulfill this criterion should be eliminated from the analysis.

Example 3

Human Genetic Analysis Using the Format-I RAA

This example illustrates the application of the method of the invention for genome-wide genetic analysis in humans. Human is an example of a high complexity genome (size ~3,000 Mb) combined with a very low level of genetic variability. Single nucleotide differences between pairs of allelic sequences from different individuals occur approximately once in every 1000 basepairs; in the population at large, the frequency may be in the order of 1:300. As with Arabidopsis, such a low frequency necessitates the use of a selection procedure for the isolation/enrichment of the rare ESP-harboring fragments. In this example a batch-wise hybridization is used to accomplish this.

Based on the known mutation frequencies, it can be estimated that the ESP frequency for a tetracutter-probing enzyme is in the order of 1 in 125 recognition sites. This low level of genetic variation, in combination with the sensitivity of micro-

array hybridization, limits the number of ESPs that can be detected in a single assay (typically ranging from a few hundred to one thousand, a few thousand at the most). These limitations can, to a certain extent, be overcome by choosing probing enzymes that recognize tetranucleotide sites containing a CpG dinucleotide. Indeed, it is well documented that a substantial fraction (≥ 25%) of the nucleotide substitutions in the human genome result from C → T transitions in CpG dinucleotides. Such CpG dinucleotides represent mutational hotspots in vertebrates because a large fraction of the cytosines are methylated and subsequently mutate to thymine by deamination. It is estimated that the mutation frequency of methylated cytosines is 6 to 8-fold higher than average. Hence probing enzymes that cleave CpG-containing recognition sites will yield ESPs at correspondingly higher frequencies, estimated at ~5%. However, the adverse consequence of the high mutation rate is that CpG is relatively rare in mammalian DNA, occurring with a frequency of 1 in 100 nucleotides [Wang, D. G. *et al.*, *Science* **280**:1077-1082 (1998)] instead of 1 in 16. Likewise the frequency of CpG-containing tetranucleotide sites is 1 in ~1600 instead of 1 in 256 bases. To compensate for this, a probe endonuclease reagent can be used, comprising of two or more of the following complementary restriction enzymes: TaqI (TCGA), MspI (CCGG), MaeII (ACGT), and HinPI or HhaI (GCGC). It should be noted however that cleavage by MaeII as well as the isoschizomers HinPI and HhaI is blocked by methylation of the cytosine residue (C^5) within the CpG dinucleotide. These enzymes will thus only cleave at a fraction of their sites, namely the non-methylated sites. Analysis of the large amount of publicly accessible human genomic DNA sequence shows that the cocktail of the 4 enzymes will cleave once in every 400 bp on average. The total number of sites in the genome is thus in the order of 7.5 million. Assuming that the ESP frequency is 5%, the enzyme cocktail has the potential of detecting ~375,000 ESPs. In addition to using combinations of restriction endonucleases, one may also use reaction conditions that decrease the cleavage specificity. Such a strategy has been applied to obtain a restriction endonuclease reagent, designated CGaseI, that is capable of cleaving DNA at CpG dinucleotides [Mead D. *et al.*, WO 94/21663]. This CGaseI restriction endonuclease reagent may be particularly useful for the analysis of human polymorphisms using the methods of the present invention.

The example described below illustrates the approach in a limited scale assay, which characterizes the human ESPs within CpG-containing tetranucleotide recognition sites using the sampling enzyme combination PacI - BfaI. The rare cutter PacI is estimated to have only about 50,000 cleavage sites in the human genome; the frequent cutter BfaI will generate two fragments per PacI site. The enzyme combination will, therefore, create a moderately complex set of 100,000 PacI-BlaI target fragments. This fragment set captures a sizable number of CpG-containing restriction sites, estimated in the order of 40,000. Assuming a 5% ESP frequency, the number of detectable ESPs is in the order of 2000. It should be stressed that many different sampling enzyme combinations can be used and that thus a substantial fraction of the ~375,000 ESPs located within NCGN-type restriction sites can be monitored.

The procedure outlined in this example comprises the following steps:

(1) Development of a set of candidate PacI-BfaI ESP fragments

(2) Genetic analysis of humans using ESP probe fragments

Step 1. Development of a set of PacI-BfaI probe fragments

A mixture of sample fragments, derived from various individuals in the population, can be divided in three classes with respect to sites for the probing enzyme: monomorphic fragments that are devoid of a cleavage site, fragments that are always cleaved, and fragments that carry one polymorphic recognition site. Fragments that are digested will be referred to as S+ fragments and fragments lacking the site as S- fragments. Polymorphic ESP fragments will thus be the only fragments present in both the S+ and S- population of sampling fragments. This forms the basis for their selection by batch-wise hybridization: only ESP fragments are capable of annealing when mixing the S+ and S- fragment collections. The hybridization-selection can be performed in two different, reciprocal ways: either the S+ fragments can be used to retrieve the matching S- fragments, or S- fragments are used to collect the complementary S+ sampling fragments. In one approach, the selected candidate ESP fragments may be isolated by cloning, arrayed, and subsequently validated by testing various sample DNAs (e.g. the various sample DNAs used as starting material for the hybridization-selection). Candidate ESP probe fragments that appear to detect

monomorphic sample fragments may either be removed from the array or retained as control elements on the array. An alternative approach consists of performing the two reciprocal hybridization-selections, cloning the selected fragments, and identification of ESPs by means of matching S+ and S- fragments. The latter strategy is outlined below.

(i) Preparation of S+ and S- fragments The preferred starting material is an equimolar mixture of genomic DNA from a number of representative individuals. Such individuals (ranging from 5 to 50) may be chosen from various CEPH (Centre d'Etude du Polymorphisme Humain) pedigrees [Wang, D. G. *et al.*, *Science* **280**:1077-1082 (1998)]. Following cleavage of the DNA mixture with the PacI/BfaI-combination of sampling enzymes, appropriate oligonucleotide adapters as described above are ligated to the fragment ends. This template DNA is divided in two aliquots and treated separately to prepare respectively the S+ and S- fragment mix. To prepare the S- fragment mix, the target DNA fragments are cleaved with the probing enzyme and then amplified. This will result in a mixture of fragments that do not contain sites for the probing enzyme. Furthermore, the S- fragment mixture may be prepared by using one biotinylated primer, such that the resulting PCR product can be captured onto a solid substrate, such as magnetic beads conjugated with streptavidin. S+ fragments are prepared by (1) amplifying the mixture of PacI-BfaI fragments, (2) digesting the PCR product with one of the four NCGN-recognizing enzymes, (3) ligating appropriate adapters to the ends generated by the probing enzyme (see EP 0 534 858, incorporated herein by reference), and (4) re-amplification of the resulting material using one primer that recognizes the probe enzyme adapter and one primer that recognizes one specific sampling enzyme adapter. Similar to the S- fragments, the amplification reaction can be performed making use of a biotinylated primer that matches the probe enzyme adaptor such that the S+ fragment mixture can be immobilized.

Two alternative pairs of PacI- and BfaI-adaptors, as well as corresponding non-selective primers are used; e.g. set I is used for the amplification of the S- fragments and set II for the preparation of S+ fragments:

Set I

5'-CTCGTAGACTGCGTACCCAT (SEQ ID NO: 17)
3'-CATCTGACGCATGGG (SEQ ID NO: 18)

5'-GACGATGAGTCCTGAG (SEQ ID NO: 3)
3'-TACTCAGGACTCAT (SEQ ID NO: 4)

5'-GACTGCGTACCCATTA (SEQ ID NO: 19)

5'-GATGAGTCCTGAGTAG (SEQ ID NO: 10)

Set II

5'-GAGCATCTGACGCATGGGAT (SEQ ID NO: 20)
3'-GTAGACTGCGTACCC (SEQ ID NO: 21)

5'-CTGCTACTCAGGACTG (SEQ ID NO: 13)
3'-ATGAGTCCTGACAT (SEQ ID NO: 14)

5'-CTGACGCATGGGATTA (SEQ ID NO: 22)

5'-CTACTCAGGACTGTAG (SEQ ID NO: 16)

The adaptor ligated to the ends generated by the NCGN-cleaving probing enzyme and the corresponding amplification primer have the following structures:

5'-GTCCTCATCGAGCATG (SEQ ID NO: 23)
3'-AGTAGCTCGTACGC (SEQ ID NO: 24)

5'-CCTCATCGAGCATGCG (SEQ ID NO: 25)

(ii) Hybridization-selection step(s) The S- fragment mix is hybridized to the biotinylated S+ fragments. Following hybridization, the biotinylated products are captured onto streptavidin-coated magnetic beads. The beads are repeatedly washed to remove all unhybridized fragments and thereafter the hybridized S- fragments are eluted. These are then reamplified with the PacI and BfaI primers and the hybridization-selection procedure is repeated at least once. Finally the amplified

fragments are cloned in an appropriate vector and a series of around 2,000 inserts are sequenced. To select a set of S+ fragments, this procedure is repeated in reverse using this time biotinylated S- fragment. Upon comparison of the S+ and S- sequences ESP fragments are readily identified as fragments having partially overlapping sequences and in which the S- fragment sequence shows a mutated NCGN restriction site at the internal boundary of the overlap. In this way, ≥500 ESPs are readily characterized.

Step 2. Genetic analysis of humans using ESP probe fragments

The sequence-verified ESP fragments are spotted on micro-arrays for genetic analysis of human sample DNA. For the preparation of this sample DNA, a pair of adaptors/primers is used that differs from those attached to the arrayed S- or S+ set of ESP fragments. From each individual, an undigested control sample and a probe enzyme digested test sample are prepared. These samples are labeled with Cy3 and Cy5, mixed and hybridized to the micro-arrays as described before. Alternatively, the hybridization mixture may be composed of differentially labeled test DNA and previously genotyped control DNA, both digested with the probing endonuclease. In both cases, the Cy3 (test/digested sample) and Cy5 (control/undigested DNA) signal intensities are normalized using a number of monomorphic control probes. The ratio of these normalized Cy3/Cy5 signals for each of the ESP probes, allows accurate determination of the allelic state of the sample at each polymorphic site (homozygous S+/S+, homozygous S-/S-, heterozygous S+/S).

The micro-array hybridization experiment may in the first place be performed with the sample DNAs, deriving from a collection of individuals, from which the ESP probe fragments were isolated. Such an experiment will, in the first place, confirm the polymorphic nature of the selected probe fragments and allow their testing in a multitude of measurements. The data will also yield information on the allele frequencies among an appreciable number of chromosomes.

Example 4

Human genetic analysis using format-II RAA

As described for corn in Example 2, the format-I ESP assay for human genetic analysis may be converted to a format-II or a format-III assay. Based on the sequence of the selected and experimentally validated ESP fragments, it is indeed possible to design a pair of dedicated, i.e. ESP-specific, PCR primers. Such primers can be combined in a number of parallel multiplex reactions, which are in turn combined to obtain the sample DNA [Wang, D. G. *et al.*, *Science* **280**: 1077-1082 (1998)]. This sample DNA is hybridized against a micro-array of spotted S+ ESP fragments (*see* to Example 3). The experiment is set up such that the fluorescently labeled ESP-specific primer and the S+ sequences are located on opposite sides of the polymorphic site. Alternatively, the unlabeled ESP-specific amplification primers may be arrayed as hybridization probes. The development of a format-II or format-III assay need not be preceded by the identification of ESP fragments (using one of the methods described in the previous examples). In the present example, we describe the development of an RAA assay based on the sequence of previously discovered SNPs.

Close inspection of the known SNPs reveals that a significant percentage of them are associated with both the loss and gain of a restriction recognition site, i.e. each of two allelic sequences is associated with a different restriction recognition site. The single nucleotide substitution may inter-convert recognition sequences that are identical except for one nucleotide [e.g. PleI (GACTC) and HgaI (GACGC), HgaI and SfaNI (GATGC), SfaNI and BbvI (GCTGC)]. Alternatively, the allelic recognition sites may be partially overlapping [e.g. MaeII (ACGTg) and NlaIII (aCATG); in the latter case the inter-conversion depends on the nature of the upstream or downstream sequences). Such mutually exclusive restriction site allelism offers a distinct advantage. The RAA technique will normally only detect the allele that is devoid of a recognition site for the probing enzyme; therefore, determination of the zygosity requires careful calibration of the signal against that observed with undigested control DNA. When each allele is associated with the presence/absence of a restriction site, two parallel RAA-assays can be performed, each involving digestion with one of the alternative enzymes.

With such an assay, both alleles can be positively identified and the zygosity is readily determined. The two parallel assays are best performed in a two-color mode; one of the primers is differentially labeled (e.g. with Cy3 and Cy5 as described previously) such that the amplification reactions can be mixed and hybridized against a single array of probes.

We have systematically explored the SNP database of the Whitehead Institute for mutational changes that promote restriction site inter-conversions and have calculated their occurrence frequency. Two SNP-associated recognition site inter-conversions were found to occur at high frequency: MaeII -> NlaIII and HgaI -> SfaNI. In both cases the mutational changes converting one site into another are C→T (or G→A) transitions occurring in CpG dinucleotides. This finding is entirely consistent with the fact that this type of mutation occurs with a 6-8 times higher frequency than other nucleotide substitutions. Based on the number of SNPs found in the Whitehead database, we estimate the total number of SNPs in the human genome for the enzyme pairs MaeII/NlaIII and HgaI/SfaNI at respectively 30,000 and 15,000. These numbers are presumably somewhat overestimated since both MaeII and HgaI are susceptible to CpG methylation. Consequently the inter-conversion can only be measured at the non-methylated sites. Therefore, in practice, RAA assays designed on the basis of sequence data should be validated on a number of test samples. Assays in which no cleavage takes place at the CpG-containing site in none of the individuals tested, should be eliminated from the RAA bi-allelic marker systems.

The foregoing examples are illustrative of the invention and are not intended to be limit the scope of the invention as set out in the claims. All of the references cited herein are incorporated by reference.

WE CLAIM:

1. A method for detecting an endonuclease site polymorphism (ESP) in DNA, the method comprising:

(a) isolating sample DNA;

(b) deriving a set of concomitantly amplifiable target DNA fragments from the sample DNA;

(c) treating the target DNA fragments obtained in step (b) with a probe restriction endonuclease reagent;

(d) amplifying the probe restriction endonuclease reagent treated target DNA fragments of step(c);

(e) analyzing the DNA of step (d) to determine which target fragments are amplified and/or which target fragments are not amplified; and wherein target DNA fragments which are amplified lack a recognition site for the probe restriction endonuclease reagent and target fragments having a recognition site for the probe restriction endonuclease reagent are not amplified.

2. The method of claim 1 the concomitantly amplifiable target DNA fragment of step (b) are derived by treatment of the sample DNA with a sampling restriction endonuclease reagent.

3. The method of claim 2 wherein the concomitantly amplifiable DNA fragments of step (b) are derived from sample DNA by treatment of the sample DNA with a first and a second restriction endonuclease reagent.

4. The method of claim 3 wherein said first restriction endonuclease reagent has a recognition sequence of six or more nucleotides and the second restriction endonuclease reagents has a recognition sequence of four or fewer nucleotides.

5. The method of claim 3 or 4 wherein said concomitantly amplifiable target DNA fragments are derived by step wise treatment of said sample DNA with the

first and the second restriction endonuclease reagents.

6. The method of claim 1 further comprising preparing of PCR primers which flank the endonuclease site polymorphism (ESP) for use in amplifying said concomitantly amplifiable target DNA fragments.

7. The method of claims 1, 2, 3, and 4 wherein the concomitantly amplifiable DNA fragments are modified by ligation of adapters to both termini of said fragments, and wherein said adaptors are capable of serving as primers for amplification.

8. The method of claim 5 wherein the concomitantly amplifiable DNA fragments are modified by ligation of adapters to both termini of said fragments, and wherein said adaptors are capable of serving as primers for amplification.

9. The method of claim 1 wherein the probe restriction endonuclease reagent of step (c) has a recognition sequence comprising six or more nucleotides.

10. The method of claim 1 wherein the probe restriction endonuclease reagent of step (c) has a recognition sequence comprising four or more nucleotides.

11. The method according to claim 1 wherein the probe restriction endonuclease of step (c) has a recognition sequence of two nucleotides.

12. The method according to claim 1 wherein the order of the steps (b) and (c) are reversed or carried out simultaneously.

13. The method according to claim 1 wherein said endonuclease site polymorphism is an alteration in a concomitantly amplifiable target fragment giving rise to a nucleotide sequence that is recognized and cut by the probe restriction endonuclease reagent .

14. The method of claim 1 wherein said site polymorphism is an alteration in the nucleotide sequence of a concomitantly amplifiable target fragment which eliminates a recognition sequence for said probe restriction endonuclease reagent.

15. The method of claims 1, 2, 3 and 4 wherein said concomitantly amplifiable DNA fragments are amplified by a polymerase chain reaction.

16. The method of claim 5 wherein said concomitantly amplifiable DNA fragments are amplified by a polymerase chain reaction.

17. The method of claim 1 wherein amplified target fragments are identified by their ability to hybridize to cognate probe DNA fragments.

18. A method for obtaining probe DNA fragments for use in detecting endonuclease site polymorphisms, the method comprising:

(a) isolating sample DNA;

(b) deriving a set of concomitantly amplifiable target DNA fragments from the sample DNA;

(c) selecting from the target DNA fragments, probe DNA fragments having an endonuclease site polymorphism (ESPs) for the probe restriction endonuclease.

19. The method of claim 17 wherein said probe DNA fragments are derived by digestion of sample DNA with one or more sampling restriction endonuclease reagents.

20. The method of claim 18 wherein probe DNA fragments are derived by digestion of a pool of sample DNAs obtained from one or more individuals of a species.

21. The method of claim 18 wherein the probe DNA fragments are derived

by digestion of a pool of sample DNAs obtained from 10 or more individuals of a species.

22. The method of claim 18 wherein the probe DNA fragments derived by digestion of a pool of sample DNAs obtained from a pool of 50 or more individuals of species.

23. The method of any one of claims 19-21 wherein said species is selected from the group consisting of procaryotic species and eucaryotic species.

24. A method for obtaining probe DNA fragments for use in detecting endonuclease site polymorphisms (ESP) comprising preparing synthetic oligonucleotides based on the nucleotide sequence of amplifiable target DNA fragments containing endonuclease site polymorphism(s).

25. A method for producing a microarray of probe DNA the method comprising:

(a) isolating sample DNA;

(b) deriving a set of concomitantly amplifiable target DNA fragments from the sample DNA;

(c) selecting probe DNA fragments having restriction endonuclease site polymorphisms (ESPs) from the sample restriction endonuclease treated target DNA fragments of step (b); and

(d) arraying the probe DNA fragments obtained in step (c) on a solid substrate in a predefined region by attaching the fragments to the substrate.

26. The method of claim 24 wherein the DNA fragments of step (b) are obtained by treating sample DNA with one or more sample restriction endonuclease reagents.

27. The method of claim 24 wherein the said probe DNA fragments of step

(d) are synthetic oligonucleotides which correspond to the concomitantly amplifiable target DNA fragments derivable from said sample DNA and containing an endonuclease site polymorphism (ESP).

28. The method of claim 25, 26 or 27 wherein the solid support is selected from a group consisting of a planar solid support, a bead, a sphere and a polyhedron.

29. The method of claim 25 wherein the microarray comprises at least 2,000 probe fragments.

30. The method of claim 26 wherein the microarray comprises at least 2,000 sythetic ologonucleotides.

31. The method of claim 27 wherein the microarray comprises at least 2,000 probe fragments.

32. The method of claim 28 wherein the microarray comprises at least 2,000 probe fragments.

33. The method of claim 25 wherein the microarray comprises at least 20,000 probe fragments.

34. The method of claim 26 wherein the microarray comprises at least 20,000 sythetic ologonucleotides.

35. The method of claim 27 wherein the microarray comprises at least 20,000 probe fragments.

36. The method of claim 28 wherein the microarray comprises at least 20,000 probe fragments.

Figure 1

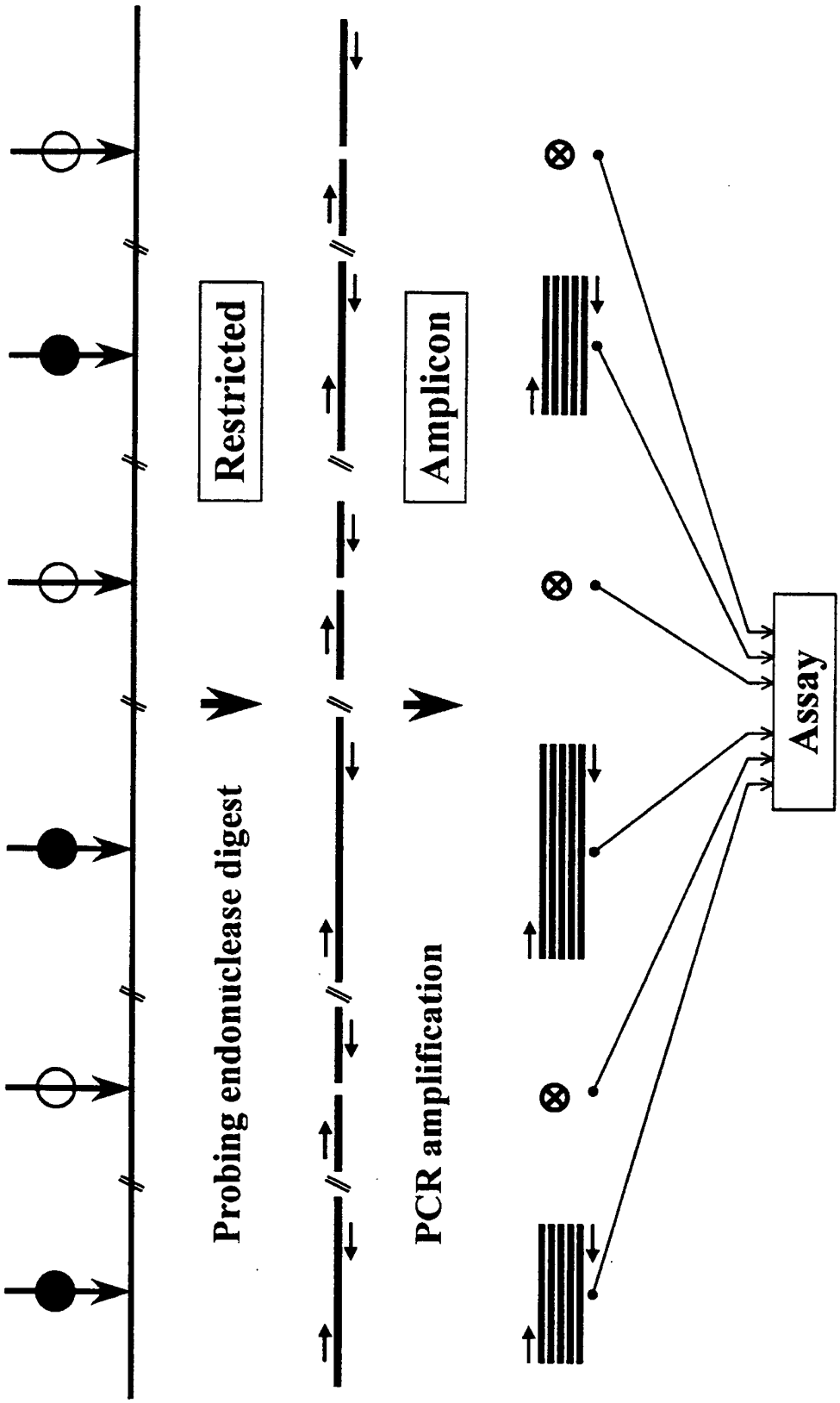

Probing endonuclease digest
Restricted
PCR amplification
Amplicon
Assay

Figure 2

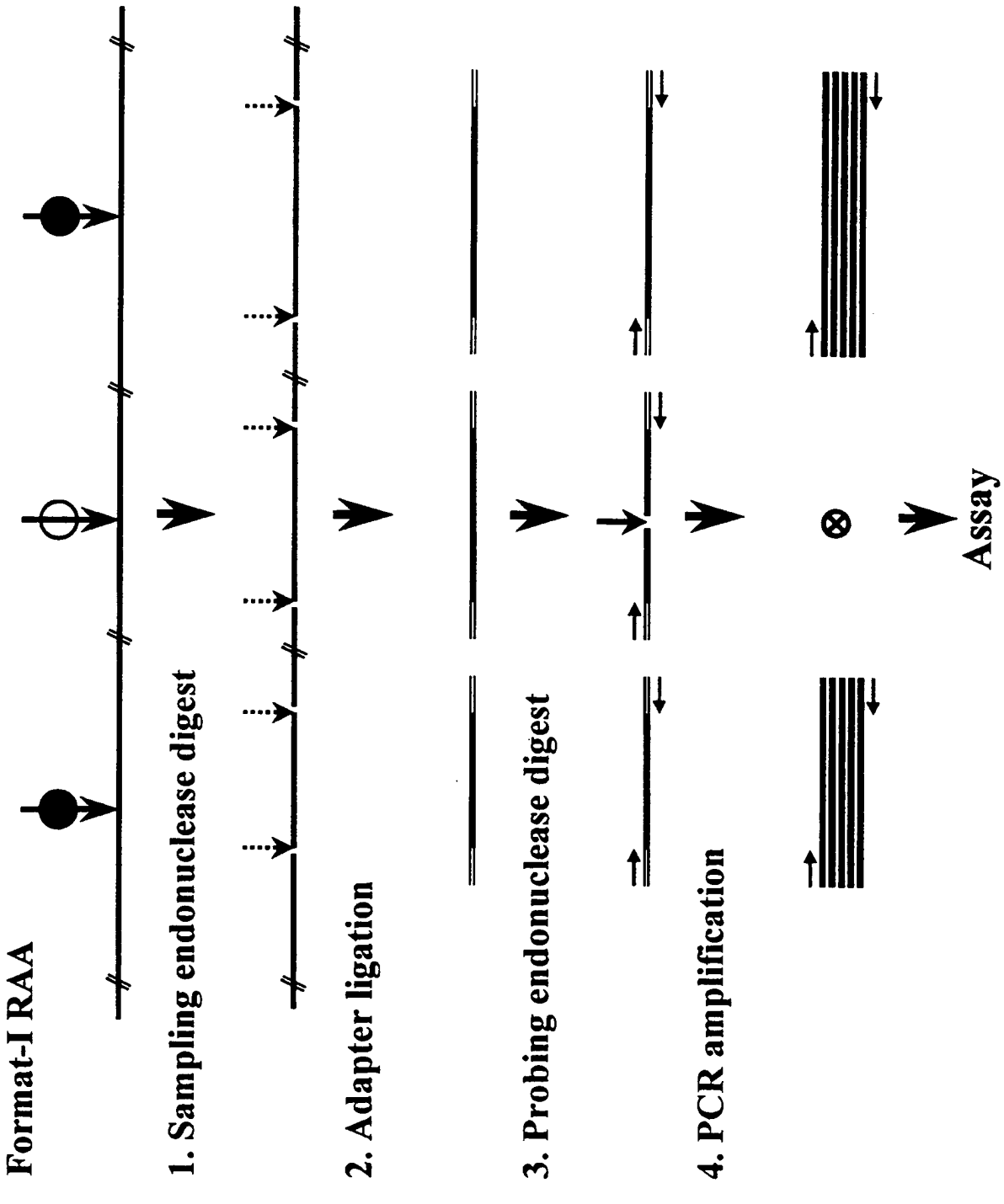

Format-I RAA
1. Sampling endonuclease digest
2. Adapter ligation
3. Probing endonuclease digest
4. PCR amplification
Assay

Figure 3

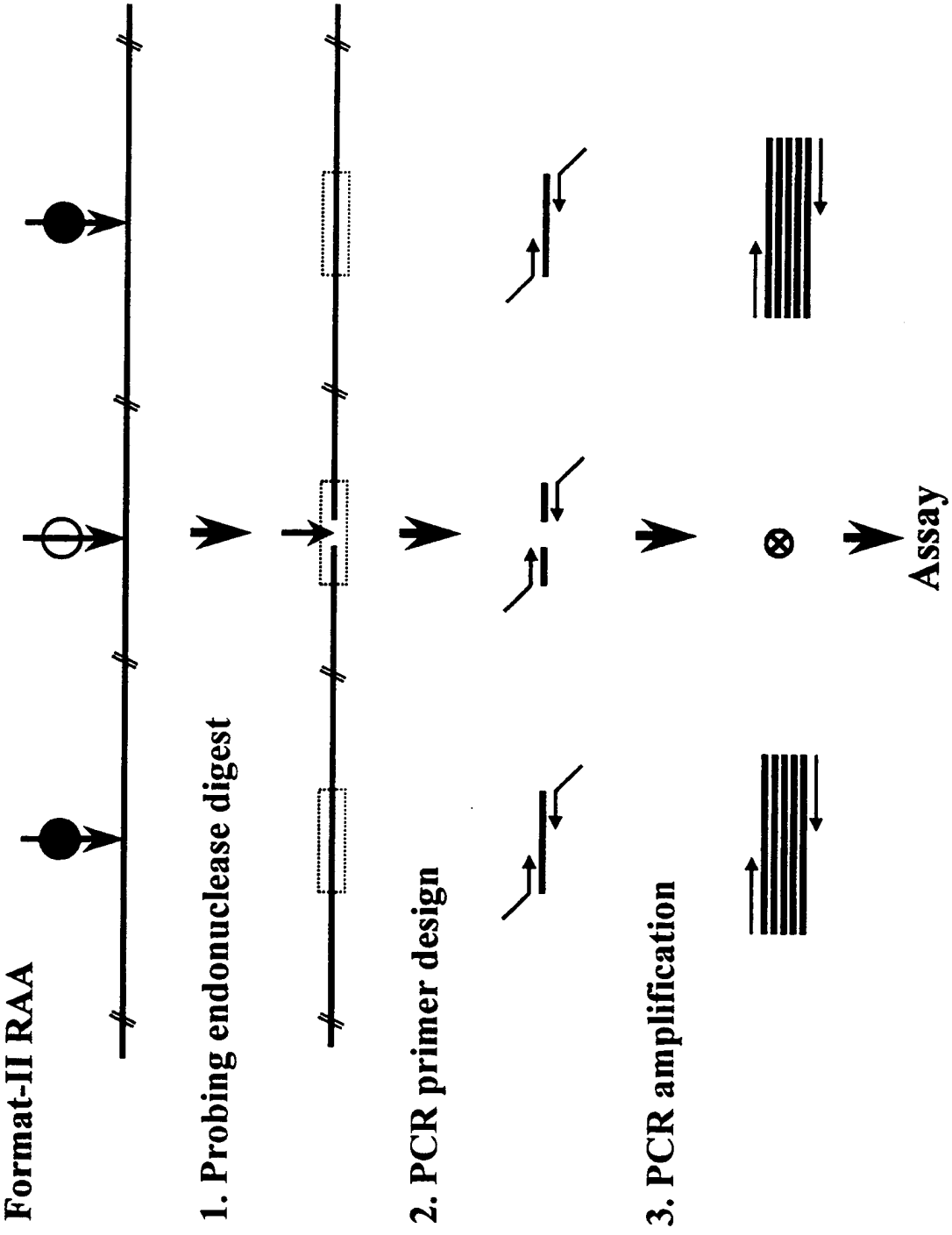

Format-II RAA
1. Probing endonuclease digest
2. PCR primer design
3. PCR amplification
Assay

Figure 4

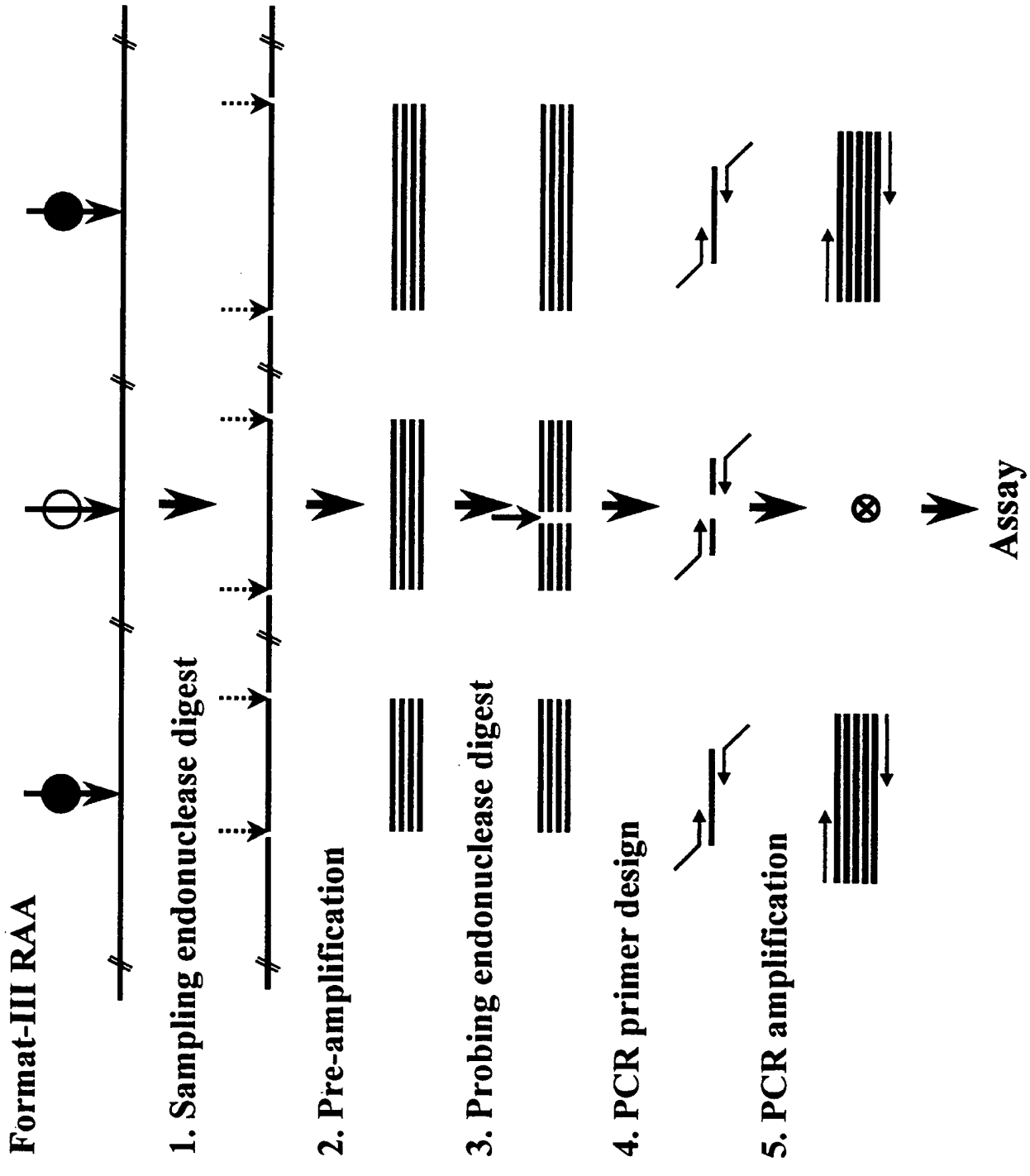

Format-III RAA
1. Sampling endonuclease digest
2. Pre-amplification
3. Probing endonuclease digest
4. PCR primer design
5. PCR amplification
Assay

Figure 5

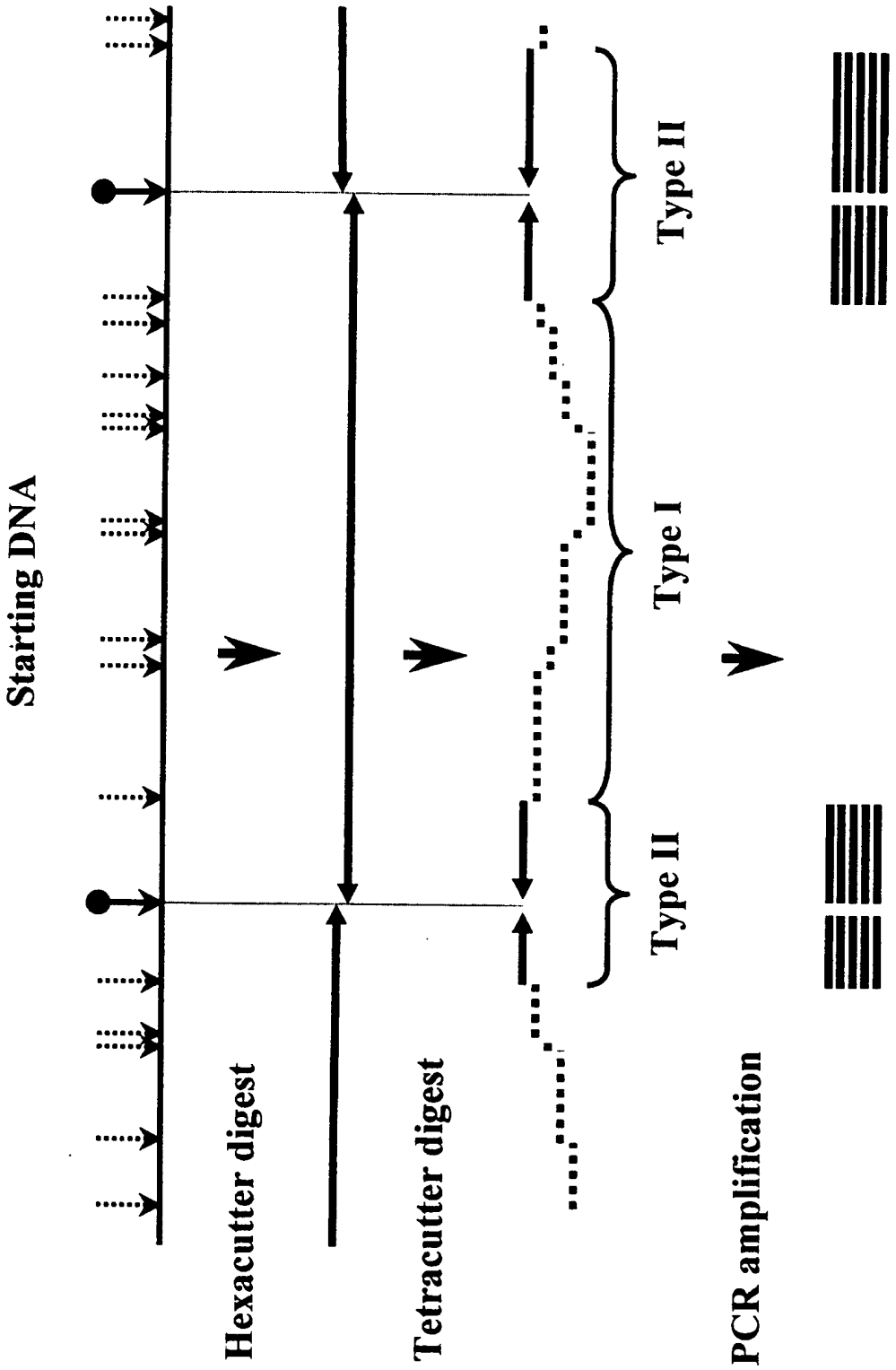
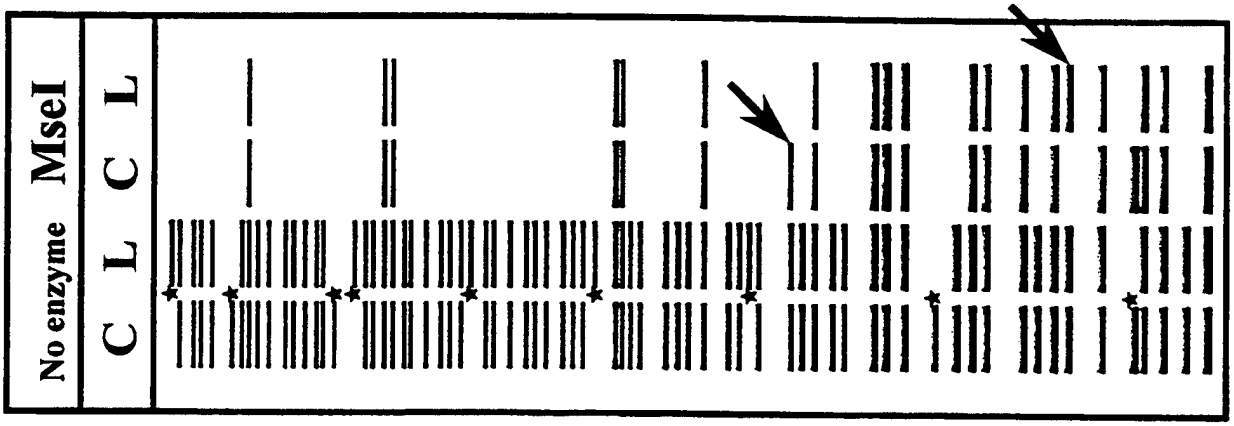

Starting DNA
Hexacutter digest
Tetracutter digest
PCR amplification
Type II
Type I
Type II

Figure 6



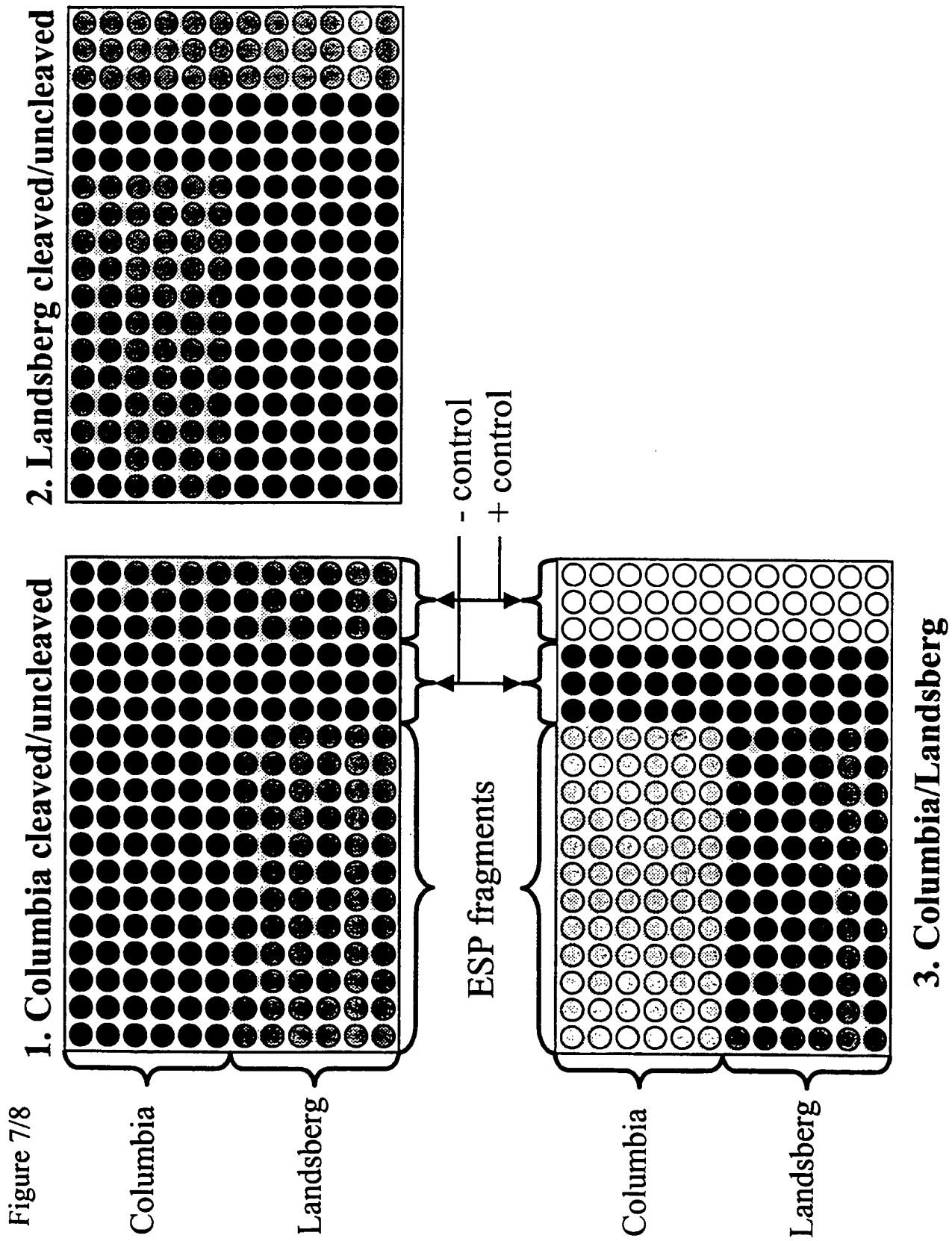
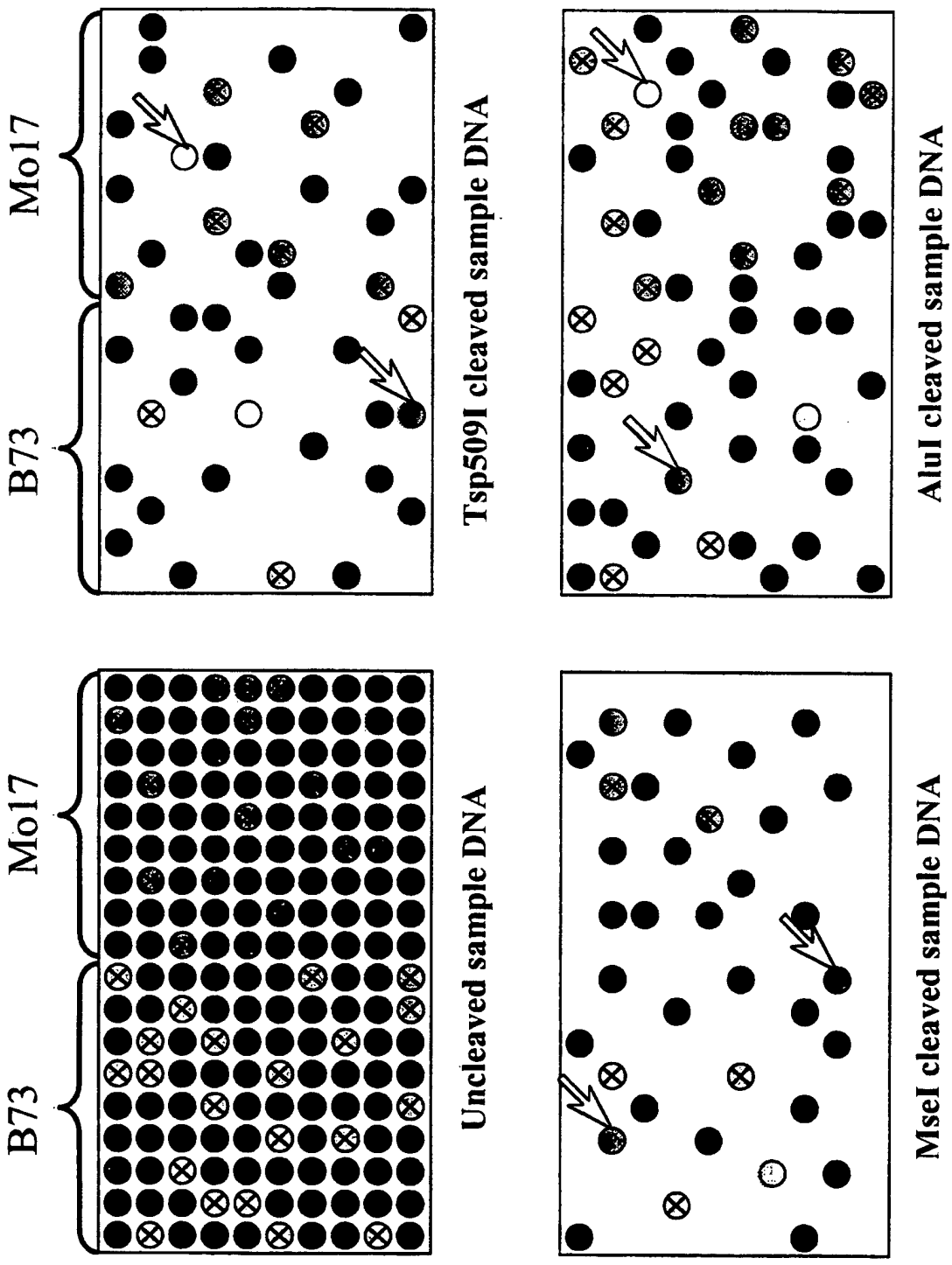

1. Columbia cleaved/uncleaved
2. Landsberg cleaved/uncleaved
- control
+ control
ESP fragments
Columbia
Landsberg
Columbia
Landsberg
3. Columbia/Landsberg

Figure 7/8

Figure 8/8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Mo17
Tsp509I cleaved sample DNA
AluI cleaved sample DNA
Uncleaved sample DNA
MseI cleaved sample DNA

SEQUENCE LISTING

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<170> PatentIn Ver. 2.0

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<212> DNA
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<400> 23
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Supplementary Material

Principal C mp nents Analysis

Although an analysis of variance (ANOVA) can be used to assess within-platform vs. between-platform variability on a gene-by-gene basis, it cannot be performed to analyze multiple genes simultaneously. For this purpose, we used a multivariate approach based on principal components analysis (PCA) (1) as visualized by Partek Pro (v. 5) software to summarize within-platform vs. between platform variability across a multidimensional scale. Like the comparison of the distribution of Z-scores, making comparisons between platforms with PCA requires that we bring all measurement technologies to a single common scale. In order to achieve this, we further applied a van der Waerden's transformation to the expression values from each chip for a direct comparison of platforms. This transformation entails replacing the data with its ranks and then applying an inverse normal transformation to the result to give (in the absence of tied values) a perfectly normal distribution. Supplemental Figure 3a shows the first two principal components of the data, which account for most of the variability in the multidimensional dataset (34.9 + 37.3 = 72.2%). Although the points (arrays) in supplemental figure 3a are color-coded by platform the principal components are computed without regard to platform category. It is clear from supplemental figure 3a that the between-platform variability is much greater than the within-platform variability (the first PC largely separating the cDNA from the two oligonucleotide platforms, and the second PC separating all platforms, especially the two oligonucleotide platforms). Supplemental figure 3a reveals that any variability due to the treatment condition is clearly overshadowed by the platform effect. Supplemental figure 3b shows a dot plot of PC #3, which clearly does capture the treatment effect. Since PC #3 explains 8.5% of the variability of the entire data, there does seem to be an ability to distinguish differential expression due to the treatment after masking out platform effects.

Contingency Table Analysis

After modeling gene expression with an ANOVA model using a .001 alpha (Supplemental Table 6), we found that the Amersham assay detected the largest number of genes (117), the Agilent assay identified 67, and the Affymetrix assay found 34 differentially expressed genes. McNemar's test statstics computed for each of the three platform pairs indicated significant differences ($p < .017$) in the number of genes found to be differentially expressed by each of the technologies. This observed difference may reflect the different levels of experimental variability associated with each of the platforms as seen in figure 3. For two of the comparisons, the Fisher's exact test for association between the gene sets were significant ($p < .017$) demonstrating that agreement between the 2 platforms occurred in a manner that was non-random. However, when comparing the Amersham and Affymetrix gene lists, no significant (non-random) association could be found.

A subset of the genes in our lists exhibited statistically significant differential expression at an alpha cut-off of .001 with less than 2-fold differential expression. Since microarray technologies are frequently considered more accurate in detecting genes differentially expressed at levels greater than or equal to 2-fold (2), we applied an additional 2-fold change (in both directions) minimum criterion upon any genes found significant at a .001 alpha level to assess whether it would increase the level of overlap of differentially expressed genes detected by each platform. Despite applying this additional fold change criterion, we could not reject the null hypothesis ($p < .017$) of no association between the Amersham and Affymetrix gene lists (Supplemental Table 7). Only when the alpha cutoff was reduced

to .01 with a 2-fold minimum criterion could we reject the null hypothesis of no association across all 2-way comparisons (Supplemental Table 8).

Supplemental Table 1: Pearson's product-moment and Spearman's rank-order correlation coefficients of gene expression measurements from 3 commercial microarray technologies matched by their Unigene ID. P-values of the hypothesis of no correlation are also reported.

Comparison	Platform A	Platform B	Pearson's	P-value	Spearman's	P-value	N
1	Amersham	Agilent	0.54505	<.0001	0.54630	<.0001	8024
2	Amersham	Affymetrix	0.59118	<.0001	0.58727	<.0001	8024
3	Agilent	Affymetrix	0.56198	<.0001	0.55232	<.0001	8024

Supplemental Table 2: Pearson's product-moment and Spearman's rank-order correlation coefficients of the fold change of time 0 hours and time 24 hours measurements matched by their Unigene ID. P-values of the hypothesis of no correlation are also reported.

Comparison	Platform A	Platform B	Pearson's	P-value	Spearman's	P-value	N
1	Amersham	Agilent	0.63374	<.0001	0.57991	<.0001	4012
2	Amersham	Affymetrix	0.56066	<.0001	0.53731	<.0001	4012
3	Agilent	Affymetrix	0.59903	<.0001	0.59549	<.0001	4012

Supplemental Table 3: Contingency tables of differential gene expression classifications from data matched by Unigene ID that was modeled with a mixed-model nested ANOVA and an alpha cut-off of .001. In each cell the frequency, row percentage, and column percentage is reported. Probability values from Fisher's Exact Test of association and McNemar's test of agreement are also reported.

Table of Amersham by Affymetrix

Amersham	Affymetrix No	Affymetrix Yes	Total
No	3693 98.48 93.78	57+ 1.52 77.03	3750
Yes	245+ 93.51 6.22	17* 6.49 22.97	262
Total	3938	74	4012

Table of Agilent by Affymetrix

Agilent	Affymetrix No	Affymetrix Yes	Total
No	3800 98.52 96.50	57+ 1.48 77.03	3857
Yes	138+ 89.03 3.50	17* 10.97 22.97	155
Total	3938	74	4012

Table of Amersham by Agilent

Amersham	Agilent No	Agilent Yes	Total
No	3650 97.33 94.63	100+ 2.67 64.52	3750
Yes	207+ 79.01 5.37	55* 20.99 35.48	262
Total	3857	155	4012

Platform A	Platform B	Exact P-value for McNemar's Test	Fisher's Exact Test P-value (Right)
Agilent	Affymetrix	<.0001	<.0001
Amersham	Affymetrix	<.0001	<.0001
Amersham	Agilent	<.0001	<.0001

* significant non-random association

+ significantly different detection rates

Supplemental Table 4: Pearson's product-moment correlation coefficients of technical and biological replicate measurements. P-values of the hypothesis of no correlation are also reported.

Comparison	Platform	Technical Replicates	P-value	Biological Replicates	P-value	N
1	Affymetrix	0.91894	<.0001	0.91255	<.0001	4018
2	Amersham	0.99259	<.0001	0.98240	<.0001	4018
3	Agilent	0.98727	<.0001	0.96435	<.0001	4018

Supplemental Table 5: Average difference of fold change measured by two platforms

Comparison	Platform A	Platform B	Mean Difference	N	Std Dev
1	Amersham	Agilent	0.0514661	2009	0.2502959
2	Agilent	Affymetrix	0.1033182	2009	0.3141808
3	Amersham	Affymetrix	0.1547843	2009	0.3347937

Supplemental Table 6: Contingency tables of differential gene expression classifications using a mixed-model nested ANOVA and an alpha cut-off of .001. In each cell the frequency, row percentage, and column percentage is reported, in that respective order. Probability values from Fisher's Exact Test of association and McNemar's test of agreement are also reported.

Table of Agilent by Affymetrix			
	Affymetrix		
Agilent	No	Yes	Total
No	1917 98.71 97.06	25+ 1.29 73.53	1942
Yes	58+ 86.57 2.94	9* 13.43 26.47	67
Total	1975	34	2009

Table of Amersham by Affymetrix			
	Affymetrix		
Amersham	No	Yes	Total
No	1863 98.47 94.33	29+ 1.53 85.29	1892
Yes	112+ 95.73 5.67	5 4.27 14.71	117
Total	1975	34	2009

Table of Amersham by Agilent			
	Agilent		
Amersham	No	Yes	Total
No	1848 97.67 95.16	44+ 2.33 65.67	1892
Yes	94+ 80.34 4.84	23* 19.66 34.33	117
Total	1942	67	2009

Platform A	Platform B	Exact P-value for McNemar's Test	Fisher's Exact Test P-value (Right)
Agilent	Affymetrix	0.0004	<.0001
Amerhsam	Affymetrix	<.0001	0.0441
Amersham	Agilent	<.0001	<.0001

* significant non-random association

+ significantly different detection rates

Supplemental Table 7: Contingency tables of differential gene expression classifications using a mixed-model nested ANOVA and an alpha cut-off of .001 and a 2-fold minimum criterion. In each cell the frequency, row percentage, and column percentage is reported, in that respective order. Probability values from Fisher's Exact Test of association and McNemar's test of agreement are also reported.

Table of Amersham by Affymetrix			
	Affymetrix		
Amersham	**No**	**Yes**	**Total**
No	1933 98.98 97.28	20+ 1.02 90.91	1953
Yes	54+ 96.43 2.72	2 3.57 9.09	56
Total	1987	22	2009

Table of Agilent by Affymetrix			
	Affymetrix		
Agilent	**No**	**Yes**	**Total**
No	1955 99.09 98.39	18 0.91 81.82	1973
Yes	32 88.89 1.61	4* 11.11 18.18	36
Total	1987	22	2009

Table of Amersham by Agilent			
	Agilent		
Amersham	**No**	**Yes**	**Total**
No	1933 98.98 97.97	20+ 1.02 55.56	1953
Yes	40+ 71.43 2.03	16* 28.57 44.44	56
Total	1973	36	2009

Platform A	Platform B	Exact P-value for McNemar's Test	Fisher's Exact Test P-value (Right)
Agilent	Affymetrix	0.0649	0.0005
Amersham	Affymetrix	<.0001	0.1236
Amersham	Agilent	0.0135	<.0001

* significant non-random association

+ significantly different detection rates

Supplemental Table 8: Contingency tables of differential gene expression classifications using a mixed-model nested ANOVA and an alpha cut-off of .01 and a 2-fold minimum criterion. In each cell the frequency, row percentage, and column percentage is reported, in that respective order. Probability values from Fisher's Exact Test of association and McNemar's test of agreement are also reported.

Table of Agilent by Affymetrix			
	Affymetrix		
Agilent	No	Yes	Total
No	1878 96.65 97.56	65 3.35 77.38	1943
Yes	47 71.21 2.44	19* 28.79 22.62	66
Total	1925	84	2009

Table of Amersham by Affymetrix			
	Affymetrix		
Amersham	No	Yes	Total
No	1794 96.66 93.19	62^{+} 3.34 73.81	1856
Yes	131^{+} 85.62 6.81	22* 14.38 26.19	153
Total	1925	84	2009

Table of Amersham by Agilent			
	Agilent		
Amersham	No	Yes	Total
No	1824 98.28 93.88	32^{+} 1.72 48.48	1856
Yes	119^{+} 77.78 6.12	34* 22.22 51.52	153
Total	1943	66	2009

Platform A	Platform B	Exact P-value for McNemar's Test	Fisher's Exact Test P-value (Right)
Agilent	Affymetrix	0.1078	<.0001
Amersham	Affymetrix	<.0001	<.0001
Amersham	Agilent	<.0001	<.0001

* significant non-random association

$^{+}$ significantly different detection rates

Supplemental Table 9: Contingency tables of differential gene expression classifications using a mixed-model nested ANOVA and an alpha cut-off of .001. The Affymetrix data was normalized using dChip. In each cell the frequency, row percentage, and column percentage is reported. Probability values from Fisher's Exact Test of association and McNemar's test of agreement are also reported.

Table of Agilent by Affymetrix

Agilent	Affymetrix No	Affymetrix Yes	Total
No	1922 98.97 97.12	20^{+} 1.03 66.67	1942
Yes	57^{+} 85.07 2.88	10* 14.93 33.33	67
Total	1979	30	2009

Table of Amersham by Affymetrix

Amersham	Affymetrix No	Affymetrix Yes	Total
No	1875 99.10 94.74	17^{+} 0.90 56.67	1892
Yes	104^{+} 88.89 5.26	13* 11.11 43.33	117
Total	1979	30	2009

Platform A	Platform B	Exact P-value for McNemar's Test	Fisher's Exact Test P-value (Right)
Agilent	Affymetrix	<.0001	<.0001
Amersham	Affymetrix	<.0001	<.0001

* significant non-random association

$^{+}$ significantly different detection rates

Supplemental Table 10: Contingency tables of differential gene expression classifications using a mixed-model nested ANOVA and an alpha cut-off of .001. The Affymetrix data was normalized using RMA. In each cell the frequency, row percentage, and column percentage is reported. Probability values from Fisher's Exact Test of association and McNemar's test of agreement are also reported.

Table of Agilent by Affymetrix

	Affymetrix		
Agilent	**No**	**Yes**	**Total**
No	1914 98.56 97.11	28+ 1.44 73.68	1942
Yes	57+ 85.07 2.89	10* 14.93 26.32	67
Total	1971	38	2009

Table of Amersham by Affymetrix

	Affymetrix		
Amersham	**No**	**Yes**	**Total**
No	1866 98.63 94.67	26+ 1.37 68.42	1892
Yes	105+ 89.74 5.33	12* 10.26 31.58	117
Total	1971	38	2009

Platform A	Platform B	Exact P-value for McNemar's Test	Fisher's Exact Test P-value (Right)
Agilent	Affymetrix	0.0022	<.0001
Amersham	Affymetrix	<.0001	<.0001

* significant non-random association

+ significantly different detection rates

Supplemental Table 11: Classifications based upon Afffymetrix data normalized with dChip, RMA, and MAS5. Contingency tables of differential gene expression classifications using a mixed-model nested ANOVA and an alpha cut-off of .001. In each cell the frequency, row percentage, and column percentage is reported. Probability values from Fisher's Exact Test of association and McNemar's test of agreement are also reported.

Table of RMA by MAS5			
	MAS5		
	No	Yes	
No	1950 98.93 98.73	21 1.07 61.76	1971
Yes	25 65.79 1.27	13* 34.21 38.24	38
Total	1975	34	2009

Table of dChip by MAS5			
	MAS5		
dChip	No	Yes	Total
No	1950 98.53 98.73	29 1.47 85.29	1979
Yes	25 83.33 1.27	5* 16.67 14.71	30
Total	1975	34	2009

Table of dChip by RMA			
	RMA		
dChip	No	Yes	Total
No	1952 98.64 99.04	27 1.36 71.05	1979
Yes	19 63.33 0.96	11* 36.67 28.95	30
Total	1971	38	2009

Platform A	Platform B	Exact P-value for McNemar's Test	Fisher's Exact Test P-value (Right)
RMA	MAS5	0.6587	<.0001
dChip	MAS5	0.6835	0.0001
dChip	RMA	0.3020	<.0001

* significant non-random association

Supplemental Figure Legends

Supplemental Figure 1: Scatter plots of log intensity values of the first and second experimental replicates.

Supplemental Figure 2: Scatter plots of log intensity values of the first and second biological replicates.

Supplemental Figure 3: Principal Components Analysis (PCA) of the data indicated that variation of signal values across microarray technologies was greater than signal variation caused by experimental treatment.